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Binding of Rat Brain Hexokinase to Recombinant Yeast Mitochondria: Effect of Environmental Factors and the Source of Porin[1]

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Heterologous binding of rat brain hexokinase to wild type, porinless, and recombinant yeast mitochondria expressing human porin was assessed, partially characterized, and compared to that in the homologous system (rat liver mitochondria). With porin-containing yeast mitochondria it is shown that (i) a significant, saturatable association occurs; (ii) its extent and apparent affinity, correlated with the origin of porin, are enhanced in the presence of dextran; (iii) the binding requires Mg ions and apparently follows a complex cooperative mechanism. This heterologous association does not seem to differ fundamentally from that in the homologous system and represents a good basis for molecular studies in yeast. With porinless yeast mitochondria, binding occurs at much lower affinity, but to many more sites per mitochondrion. The results indicating a major but not exclusive role for porin in the binding are discussed in terms of (i) the mode and mechanism of binding, and (ii) the suitability of the rat hexokinase–yeast mitochondria couple for the study of heterogeneous catalysis in reconstituted cellular model systems.

KEY WORDS: Mitochondria; rat brain hexokinase; porin; VDAC; yeast; cooperative binding.

INTRODUCTION

Structural organization plays an important role in the control of metabolism. Biological catalytic systems evolved to either overcome—or take advantage of—physical limitations imposed by the intricate and concentrated cellular environment. The binding of hexokinase to mitochondria in mammalian brain represents a classical example for dynamic cellular organization and rearrangement according to metabolic needs (Brdiczka, 1994; Bustamente *et al.*, 1981; Inui and Ishibashi, 1979; McCabe, 1994). It is commonly accepted that bound hexokinase has exclusive access to mitochondrial ATP, and may in turn efficiently recycle limiting ADP to oxidative phosphorylation in the matrix (Arora and Pedersen, 1988; Beltran del Rio and Wilson, 1992; Laterveer *et al.*, 1994; Saks *et al.*, 1993). The reversible association between homologous purified components has been demonstrated and characterized *in vitro* (Gellerich *et al.*, 1993; Kabir and Wilson, 1993; Polakis and Wilson, 1985; White and Wilson, 1989; Wicker *et al.*, 1993; Xie and Wilson, 1988).

Rat brain hexokinase (type I, 110 kDa) is composed of two distinct homologous domains, both structurally similar to yeast hexokinase (55 kDa), with the catalytic activity confined to the C-terminal domain (White and Wilson, 1989). Unlike in yeast, the mammalian enzyme is sensitive to allosteric inhibition by glucose-6-phosphate, and binds to mitochondria. The current model (Wilson, 1995) for its association with the outer membrane involves primarily an N-terminal hydrophobic peptide inserted in the membrane (Xie and Wilson, 1988), oligomerization of the enzyme (Xie and Wilson, 1990), and stabilization through Mg bridges between the N-terminal domain and the nega-

[1] Dedicated to the memory of Prof. Noun Shavit (deceased June 19, 1997).

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tive surface of mitochondria (Felgner and Wilson, 1977). The principal outer membrane protein counterpart in the association has been identified as the mitochondrial pore or porin (Felgner *et al.*, 1979; Nakashima *et al.*, 1986).

Porins (or VDAC[3]) are found in all eukaryotic cells where they form large hydrophilic pores in membranes (Benz, 1994). Despite the lack of significant sequence homology, their function and properties as a pore have been strikingly conserved (Forte *et al.*, 1987). It is commonly accepted that they share the same general tertiary structure (12–16 strands transmembrane β-barrel) (Mannella *et al.*, 1992; Rauch and Moran, 1994), although the exact arrangement of the pore in the membrane is yet a matter of controversy (De Pinto *et al.*, 1991; Forte *et al.*, 1987).

A dynamic structure has been proposed for mitochondrial topology, in which localized contact between inner and outer membranes (Kottke *et al.*, 1988) provides a microcompartment involved in nucleotide exchange between the matrix and cytoplasm (Brdiczka, 1994; Nicolay *et al.*, 1990). Isolated mitochondria contain fewer contact sites, but their frequency may be restored to the native state in the presence of macromolecules (Laterveer *et al.*, 1995; Wicker *et al.*, 1993). Contact sites are enriched in porin and exhibit relatively high hexokinase binding ability (Kottke *et al.*, 1988; Wicker *et al.*, 1993).

Genetic alteration of recombinant rHK (Arora *et al.*, 1993; Tsai and Wilson, 1995) have provided important information in structure–function studies of the mammalian enzyme. Similar studies of cloned VDAC in yeast (Blachly-Dyson *et al.*, 1990, 1993) helped in the molecular characterization of the assembly and function of the mitochondrial pore. The latter approach may in principle be used to clarify the mode of interaction between hexokinase and porin. However, the recombinant porin being expressed in yeast, binding studies to altered porin are limited to heterologous systems only (mammalian hexokinase and yeast mitochondria). In view of the probable contribution of additional, species-specific (Kabir and Wilson, 1993) microenvironmental factors in the association, the relevance of such studies in heterologous systems has been questioned (Wilson, 1997). In search of a versatile model system suited to genetic manipulation, we initiated the characterization of hexokinase binding to yeast mitochondria harboring porin from variable source. Experimental evidence obtained in heterologous systems (a preliminary report of which has been presented elsewhere, see Azoulay and Aflalo, 1996) shows no qualitative difference with the homologous one, indicating a similar condition for rat brain hexokinase binding to rat or yeast mitochondria–porin pairs.

[3] Abbreviations: BSA: bovine serum albumin; DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid; FL: firefly luciferase; G6P: glucose-6-phosphate; G6PDH: lucose-6-phosphate dehydrogenase; Hepes: 2-[4-(2-hydroxyethyl-1-piperazine)] ethanesulfonic acid; PMSF: phenylmethylsulfonyl fluoride; rHK: rat brain hexokinase; R+: rat liver mitochondria; U: unit of enzyme activity (= 1 μmol product/min); VDAC: voltage-dependent anion channel; Y+: wild type yeast mitochondria; Y−: porinless yeast mitochondria; Yh′: yeast mitochondria expressing recombinant tagged human porin; yHK: yeast hexokinase.

MATERIALS AND METHODS

Baker's yeast hexokinase (yHK), bovine pancreas trypsin, soybean trypsin inhibitor, and *Leuconostoc* glucose-6-phosphate dehydrogenase (G6PDH), as well as ATP, ADP, NAD^+, dextran (average M_r = 40,000), and cibacron blue-agarose were from commercial sources.

Biological Materials

Adult white rats (var. Sprague–Dawley) were the source of brains and livers for hexokinase (rHK) and mitochondria (R+) preparations, respectively. Yeast (*S. cereviciae*) strains DL1 (Hase *et al.*, 1984) and the mutant M22-2 (Blachly-Dyson *et al.*, 1990) were used for wild type (Y+) and porinless (Y−) mitochondria preparations, respectively. Yeast mitochondria harboring tagged human porin (Yh′) were isolated from the M22-2 strain expressing the recombinant human porin gene HVDAC1-HA (with a short hemagglutinin tag at the C-terminus) (Yu *et al.*, 1995).

Preparation of Mitochondria

Rat liver mitochondria were isolated according to published procedures (Hovious *et al.*, 1990). After homogenization of rat liver, the mitochondria were isolated in isosmotic buffer (0.3 M sorbitol, 50 mM K-Hepes pH 7.8, 1 mM PMSF, and 1 mg/ml BSA) by three cycles of differential centrifugation, and purified on Percoll (30% in buffer above) density gradient centrifugation (90,000*g*, 60 min). The final mitochondrial pellet was resuspended in 0.3 M sorbitol to give a

protein concentration of 20 mg/ml, and stored at −70°C.

Yeast mitochondria were isolated from exponential cultures growing aerobically on selective medium containing lactate as described (Aflalo, 1990; Hase *et al.*, 1984). Briefly, yeast spheroplasts were prepared and homogenized, and mitochondria isolated by three cycles of differential centrifugation in an isosmotic buffer (Buffer A: 0.6 M sorbitol, 50 mM K-Hepes pH 7.8, 20 mM KCl) supplemented with protective reagents (1 mM DTT, 1 mM PMSF, 1 mM K-EDTA, and 1 mg/ml BSA). The final mitochondrial pellet was resuspended in Buffer A at a protein concentration of 20 mg/ml and used immediately or rapidly frozen and stored at −70°C for up to one month with some loss of phosphorylative activity (20–30%). Protein was determined by the biuret method using BSA as a standard. This preparation yields rather pure mitochondria substantially free of microsomal contamination, as assessed by electron microscopy. Further purification on Percoll gradient did not affect phosphorylative or hexokinase binding activities.

Rat Brain Hexokinase Preparation

Rat brain hexokinase was prepared according to a procedure developed by Wilson (1989), involving the isolation of crude brain mitochondria including the tightly bound enzyme. Incubation with 1.2 mM G6P and 0.5 mM K-EDTA releases the enzyme to the soluble fraction. Further purification of the enzyme in solution is achieved by affinity chromatography on cibacron blue-agarose (Pharmacia), from which hexokinase is specifically eluted by G6P. The purified enzyme was concentrated and washed in 10 mM K-Hepes pH 7.8, 0.5 mM K-EDTA, 10 mM glucose, and 1 mM DTT, using an Amicon device and a 50,000 molecular weight cut-off membrane (Spectrum, type C). Protein was determined according to Bradford (1976) using ovalbumin as a standard. The concentrated enzyme (up to 1 mg/ml, 30–60 U/mg) was frozen in liquid nitrogen and stored at −70°C until use (up to 6 months) with no apparent loss of catalytic nor rat liver mitochondria-binding activity (75–90% bindable).

Spectrophotometric Assay of Hexokinase Activity

Hexokinase activity was measured spectrophotometrically at room temperature, by coupling NADH formation by G6PDH to G6P production by hexokinase. The reaction mix (1 ml) contained 20 mM K-Hepes pH 7.8, 4 mM Mg-Hepes, 1 mM K-EDTA, 0.6 mM NAD^+, 10 mM glucose, 1 mM ATP, 1 mg/ml BSA, and 0.5 U of G6PDH. Aliquots containing hexokinase in the range 0.5–5 mU were added to the mix, and the steady-state rate of absorbance increase at 340 nm was recorded and used to calculate hexokinase activity. Under these conditions a linear dose response was obtained in the assay.

Standard Protocol for Binding of Hexokinase to Mitochondria

Yeast or rat brain hexokinase (250 mU/ml) was incubated with yeast or rat liver mitochondria (2 mg/ml) in a reaction mix (0.1 ml) containing 5 mM Mg-Hepes pH 7.8, 1 mg/ml BSA, and sorbitol at isosmotic concentration (0.6 M or 0.3 M, respectively). Where indicated, dextran (routinely 25% w/v, equivalent to 6 mOsm) was added to the incubations. After reaching equilibrium (one hour on ice), the mixture was centrifuged (13,000*g* for 10 min) to separate between free (supernatant) and bound (pellet) hexokinase fractions. After solubilization of the pellet (0.1 ml of 2% Triton X-100, 2 mM K-EDTA, and 10 mM glucose), aliquots of bound and free fractions were analyzed for hexokinase activity. Control experiments indicated that the conditions for measurement of the activity in the pellet (presence of mitochondria solubilized in detergent) or in the supernatant (presence of dextran) had no effect on the determination. Moreover, no hexokinase-like activity was detectable in mitochondrial supernatants or solubilized pellets after incubation without added hexokinase.

The fraction of bound hexokinase is routinely expressed as the percentage of the activity of the bound fraction, relative to the sum of these found in the bound and free fractions. The latter was commonly found in the range of 95–105% of the activity added at the beginning of the experiment.

RESULTS

In order to characterize the optimal binding conditions for the heterologous system composed of brain hexokinase (rHK) and yeast mitochondria, we started with the basic conditions described in Materials and Methods including minor modifications to the proce-

dure initially developed for binding rHK to rat mitochondria (R+) (Rose and Warms, 1967; Xie and Wilson, 1990), in which Mg^{2+} and a large excess of brain or liver mitochondria over hexokinase was used in the binding mix. Yeast hexokinase (yHK), a nonbindable species (Kovac *et al.*, 1986; Wilson, 1997), was used as a negative control to detect nonspecific adsorption to mitochondria. Finally as a positive control, we used rat liver mitochondria (R+) which efficiently bind purified rat brain hexokinase in these conditions.

Effect of Macromolecules

In the case of rHK binding to yeast mitochondria, a lower propensity for association between the heterologous components is anticipated *a priori*. This could in principle be overcome by increasing their concentrations. By using dextran, a molecular crowding agent which reduces the chemical activity of water in solution, the effective concentrations of both components should increase (Minton, 1993). Macromolecules have also been proposed to induce structural changes in mammalian mitochondria which were shown to be favorable for hexokinase binding (Wicker *et al.*, 1993).

The effect of dextran on binding rHK or yHK to Y+ mitochondria is presented in Fig. 1. The results indicate that only very low amounts of yHK associate with mitochondria, independently of the presence of dextran. A weak but significant association of rHK to yeast mitochondria is observed after incubation in isosmotic medium only. This binding is further greatly enhanced in the presence of up to 25% dextran in the incubation mix, as shown by the transfer of activity from solution to the mitochondrial fraction. Higher concentrations of dextran could not be used since they interfere with the isolation of mitochondria by centrifugation.

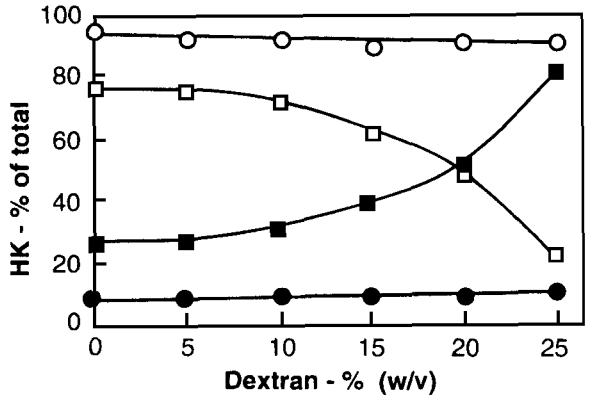

Fig. 1. Effect of dextran on the association between hexokinases and wild type yeast mitochondria. Yeast hexokinase (280 mU/ml, circles) or rat brain hexokinase (290 mU/ml, squares) were incubated with Y+ mitochondria (2 mg/ml) in the presence of dextran as indicated. Hexokinase activities in the free and bound fractions (open and closed symbols, respectively), were measured as described in Materials and Methods.

Hexokinase Binding to Different Mitochondria

In order to assess the contribution of porin in the interaction between mitochondria and rHK, the ability of mitochondria harboring porins of different sources (see Materials and Methods) to bind rHK was tested. The results of several binding experiments are summarized in Table I.

The enzyme from yeast binds or adsorbs significantly to neither rat nor yeast organelles, even in the presence of dextran, although a slight preference toward wild type yeast mitochondria (Y+) can be evoked. The affinity for association of the rat brain enzyme with different mitochondria should be reflected in the binding observed at limiting rHK ligand concentrations in the absence of dextran. As shown in Table I, the binding of rHK decreases in the order R+, Yh', Y+, Y−. Rat mitochondria, containing the natural receptor for the enzyme, achieve the most effective binding, while almost no association to porinless yeast mitochondria is observed.

In the presence of dextran, all mitochondria bind rHK to the same higher extent approaching the theoretical maximum, the enhancement being inversely related to the extent of binding in its absence. This result suggests that due to macromolecular crowding both the mitochondrial binding sites and the rHK ligand have achieved effective concentrations much higher than the dissociation constant, so that most of the hexokinase added at limiting concentration associates with mitochondria independently of their source or porin content.

In these conditions, the binding competency of rHK to mitochondria was found rather variable in experiments performed with different enzyme preparations. The maximal extent of binding can be determined using a limiting amount of rat brain hexokinase and increasing concentrations of mitochondria. Figure 2 presents the results of typical experiments using different mitochondrial preparations. In the absence

Table I. Binding of Yeast or Rat Brain Hexokinase to Mitochondria with Different Porin Content[a]

Mitochondria	(porin)	yHK bound		rHK bound	
		− dextran	+ dextran	− dextran	+ dextran
		% of total ± sem (*n*)		% of total ± sem (*n*)	
R+	(rat)	4.3 ± 2.2 (3)	5.7 ± 1.7 (3)	65.1 ± 3.8 (10)	80.1 ± 1.5 (11)
Yh′	(human)	5.4 ± 2.9 (3)	6.1 ± 2.6 (3)	25.0 ± 2.9 (6)	77.0 ± 3.0 (7)
Y+	(yeast)	7.0 ± 2.9 (3)	8.4 ± 2.4 (3)	15.1 ± 2.1 (9)	75.9 ± 2.1 (10)
Y−	(none)	3.8 ± 1.5 (3)	4.6 ± 1.6 (3)	10.6 ± 1.9 (7)	72.8 ± 1.8 (8)

[a] Mitochondria (2 mg/ml) with the indicated porin were incubated as described in Materials and Methods with the indicated hexokinase (220–350 mU/ml) in the absence or presence of 25% dextran. The average and standard error of the mean (sem) for relative binding in several (*n*) experiments with different preparations of mitochondria and rHK are shown. The binding of rHK in the absence of dextran was significantly different among all mitochondria tested, and significantly different from that of the corresponding yHK binding as well.

of dextran (Fig. 2A), maximal binding of low rHK concentrations is achieved with the addition of modest amounts of rat liver (R+), relatively to the yeast (Yh′, Y+, and Y−) mitochondria, to which only a poor association is observed, corroborating the differences in affinity suggested in the screening experiments in Table I. In the presence of dextran, the binding curves are strongly shifted to the left, as if the effective concentration of mitochondrial binding sites (relative to that of limiting rHK) has increased by a factor ranging from 2 (R+) to more than 10 (Y−). While a distinct difference is still observed between rat and yeast mitochondria, rHK associates with all organelles to the same extent reached with excess rat mitochondria in the absence of dextran. At this point, only about 80% of the hexokinase activity binds to mitochondria in these experiments. Failure to reach complete binding may be due to partial degradation of rHK in which the labile N-terminal tail (Polakis and Wilson, 1985) or regulatory (bindable) domain has been separated from the catalytically active C-terminal domain. Low M_r degradation products were indeed detected on Western blots, in correlation with poor binding activity in some rHK preparations. Moreover, control experiments have shown that the fraction of hexokinase activity remaining in solution after maximal binding has been reached (in excess of mitochondria) is unable to associate with fresh mitochondria under standard conditions.

Saturation of Mitochondria with rHK

In order to determine the binding capacity for rHK of the four different mitochondrial preparations, the reciprocal experiment was done: a fixed amount of mitochondria was incubated with increasing concentrations of rHK. At high concentration of rat liver

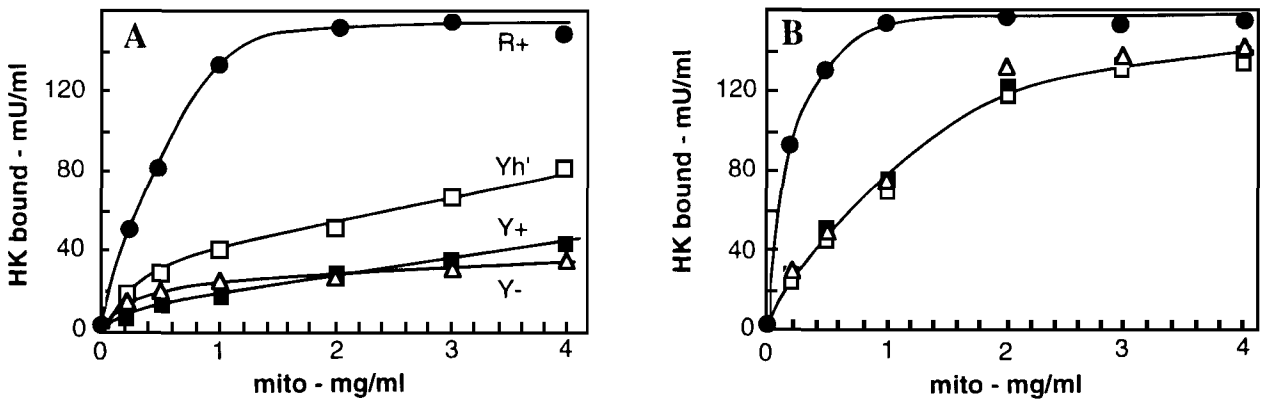

Fig. 2. Dependence of binding on mitochondria concentration at limiting hexokinase concentration. Rat brain hexokinase (190 mU/ml) was incubated with mitochondria from R+, Yh′, Y+, or Y− at the indicated concentrations as described in Materials and Methods. The same preparations of mitochondria and rat brain hexokinase were incubated in the absence (A) or presence (B) of 25% dextran.

mitochondria, maximal binding of rHK (linear increase) occurs and no saturation can be reached upon addition of rather high hexokinase concentrations in the presence of dextran (Azoulay and Aflalo, 1996). This behavior, together with the results of Fig. 2, indicates that under these conditions, all available and bindable rHK becomes tightly bound.

The experiment was thus repeated at a much lower concentration of mitochondria. In view of the significant fraction of hexokinase bound at low concentrations, the data were plotted as bound rHK vs. free rHK (Fig. 3). The porinless mitochondria bind hexokinase in two phases in the absence of dextran. At low rHK concentrations, a first pool of binding sites gets saturated, followed by a linear dependence on rHK concentration up to values exceeding those reached with porin-containing mitochondria at saturation. In the presence of dextran, the different species of binding sites (saturatable and others) become indistinguishable, and the dependence on rHK concentration appears to be linear. On the other hand, the results indicate that all the porin-containing mitochondria (i.e., R+, Yh′, and Y+) are saturatable by rHK both in the absence (Fig. 3A) and presence of 25% dextran (Fig. 3B). Preliminary analysis indicates that porin was found to be quantitatively extractable (Linden *et al.*, 1984) from wild type (R+ and Y+, 170 and 83 pmol/mg protein, respectively), but not from recombinant mitochondria (Yh′ and Y−), the latter yielding no extractable porin by this method. While the reason for the lack of recovery of HVDAC1-HA protein from Yh′ remains to be clarified, it is worthwhile noting that the maximal binding capacity of R+ and Y+ is roughly correlated with their respective porin content. The mitochondrial preparations can be characterized by a maximal rHK binding capacity (B_{max}), and the concentration of rHK needed to reach half saturation ($K_{0.5}$). The latter, while reflecting the true dissociation constant, should not be considered as a direct estimate of it, in view of the possibility of a complex mechanism of association.

Hill analysis of the rHK binding data (Fig. 4) yielded biphasic curves with a low Hill coefficient

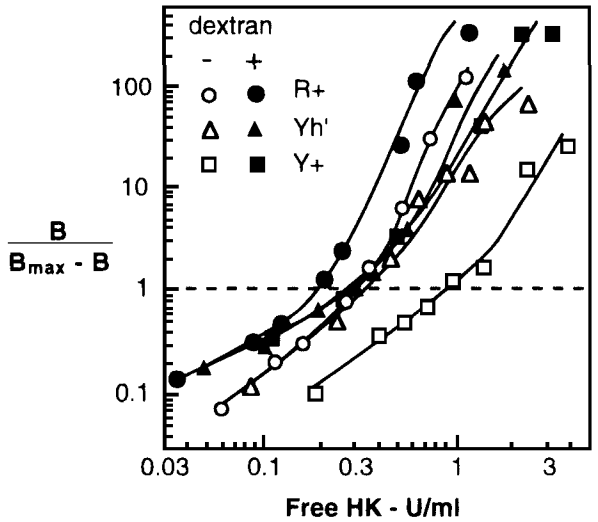

Fig. 4. Hill analysis of rHK binding to limiting amounts of mitochondria. The data in Fig. 3 were processed and replotted as the ratio of occupied to free binding sites ($B/B_{max} - B$) vs. free rHK concentrations. The values for B_{max} (mU/ml) used in the calculations were [243, 343], [132, 278], and [125, 157] for R+, Yh′, and Y+ in the absence or presence of 25% dextran, respectively. Half saturation of the mitochondria is indicated by a dashed line (ordinate = 1).

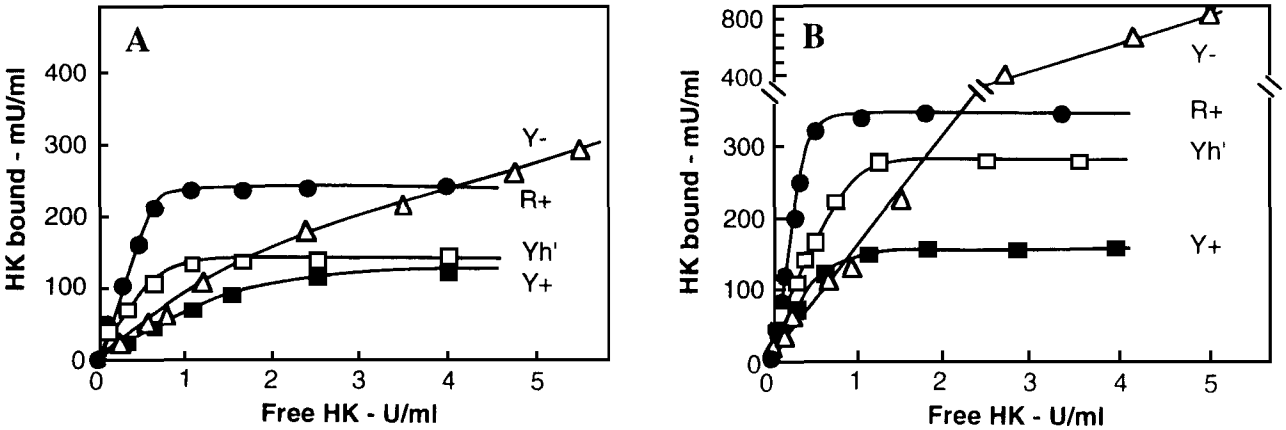

Fig. 3. Saturation of limiting amounts of mitochondria with rat brain hexokinase. Mitochondria (0.2 mg/ml) from R+, Yh′, Y+, or Y− were incubated with rHK at increasing concentrations in the absence (A) or presence (B) of 25% dextran as described in Materials and Methods. The plots represent the measured activity of rHK in the pellet (bound) as a function of that in the supernatant (free).

(1.0–1.5) in the low rHK range, and higher (2.5–4) in the high rHK range for all mitochondria. This behavior indicates that the mechanism of association is more complex than simple cooperative binding (yielding a linear Hill plot). At limiting added rHK (up to 1–3-fold in excess of B_{max}), the low apparent Hill coefficient is further reduced (from 1.5 to 1) in the presence of dextran, suggesting that in these conditions, tight binding of rHK occurs noncooperatively to independent binding sites. However, in the presence of a relative excess of rHK ligand, the high Hill coefficient (positive cooperativity in binding) is increased in the presence of dextran (see Table II). This result supports the notion of rHK being bound in an oligomeric form by a cooperative mechanism (Wicker *et al.*, 1993), as previously suggested for rat liver mitochondria in cross-linking studies (Xie and Wilson, 1990).

The results for the various porin-containing mitochondrial preparations are summarized in Table II. In the absence of dextran, $K_{0.5}$ increases in the order R+, Yh′, Y+, while the corresponding binding capacity (B_{max}) decreases in the same order. In the presence of dextran, the apparent affinity to rHK increases to reach a similar value (low $K_{0.5}$), close to that observed in the homologous system (rHK:R+) without dextran. This trend is expected, independently of the mechanism for binding, as a general effect of macromolecular crowding (Minton, 1993). Theoretical studies (based on empirical measurements) predict at 25% dextran an increase in the effective concentration of a large protein by about one order of magnitude (Minton, 1983). However, while a large increase (ca. 3-fold) in apparent affinity is observed with wild type yeast mitochondria (Y+), it is only moderate (1.3-fold) with mitochondria bearing mammalian porin (R+ and Yh′). Moreover, the neutral polymer enhances the binding capacity (B_{max}) of all mitochondria, more significantly for the latter (R+ and Yh′) as compared to wild type yeast (Y+). The apparent Hill coefficient (n_H) observed at high [rHK] in the absence of dextran is substantially lower with yeast mitochondria (Y+ and Yh′). In this respect, a distinct effect of dextran is observed in tightening the cooperativity of binding to yeast mitochondria, with the value of n_H determined for the heterologous couples rHK:Y+ and rHK:Yh′ raised to that observed for the homologous couple rHK:R+.

Formation and Stability of the Mitochondria:rHK Complexes

The effect of G6P and EDTA on the binding of rHK to mitochondria, as well as their influence on the stability of a preformed complex were tested in the absence and presence of dextran for both yeast and rat mitochondria.

A straightforward comparison of the requirements for binding rHK to rat liver (R+) vs. wild type yeast (Y+) mitochondria is presented in Fig. 5. Besides the selective enhancement by dextran observed with Y+, the presence of divalent cations (either endogenous, removable by EDTA, or added) appears to be a common and absolute requisite for binding under all conditions tested. On the other hand, no significant effect of G6P on binding to any mitochondria is detected. Moreover, the addition of the hexose-phosphate did not strengthen the requirement for Mg in binding (in some cases the effect of EDTA was somewhat attenuated). Thus the three factors tested (dextran, Mg, and G6P) appear to operate independently of the binding process with both yeast and rat mitochondria.

A similar comparison for the stability of preformed complexes between rHK and different mito-

Table II. Binding Parameters for the Association of Hexokinase to Different Mitochondria[d]

	− dextran			+ dextran		
Mitochondria	B_{max}[a] (mU/ml)	$K_{0.5}$[b] (mU/ml)	n_H[c]	B_{max}[a] (mU/ml)	$K_{0.5}$[b] (mU/ml)	n_H[c]
R+	192 ± 35	261 ± 25	4.0 ± 0.2	328 ± 18	206 ± 29	4.0 ± 0.2
Yh′	138 ± 3	341 ± 2	2.6 ± 0.3	227 ± 36	268 ± 21	4.0 ± 0.3
Y+	123 ± 2	940 ± 83	2.7 ± 0.2	163 ± 4	288 ± 28	3.5 ± 0.2

[a] B_{max} is the value for the concentration of mitochondrial binding sites in a 0.2 mg/ml suspension expressed in bound rHK equivalents (mU/ml).
[b] $K_{0.5}$ is the concentration of free rHK (mU/ml) at which half saturation of the mitochondria is observed.
[c] n_H represents the apparent Hill coefficient, calculated as the slope of Hill plots in the high rHK concentration range.
[d] The values, derived graphically from independent data sets (2–3 for each type of mitochondria) similar to those in Fig. 3 (B_{max}, $K_{0.5}$) or Fig. 4 (n_H), are presented as averages with standard errors of the mean.

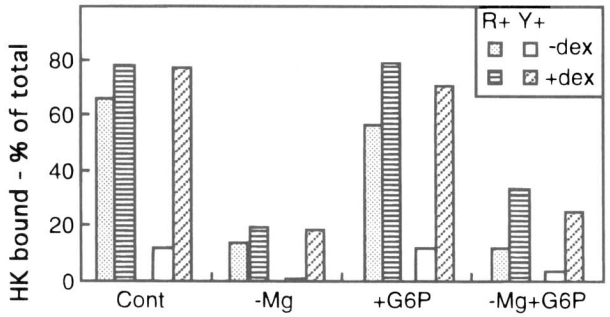

Fig. 5. Effect of dextran, Mg, and G6P on the formation of the complex mitochondria–rHK. Purified rHK (250 mU/ml, 80% bindable) was incubated in dextran (25%) as indicated with mitochondria (2 mg/ml) from R+ (left pair) or Y+ (right pair) in the standard conditions (Cont); with Mg^{2+} replaced by K^+ and 0.5 mM K-EDTA added (−Mg); with 1.5 mM G6P added (+G6P); or in a combination of both the latter conditions (−Mg+G6P). After centrifugation, the mitochondrial fraction was analyzed for bound rHK as described in Materials and Methods. The results are expressed as percentage of total rHK found in the bound fraction.

chondria under the conditions stated above is presented in Fig. 6. A major difference with *de novo* binding is observed with rat mitochondria in the absence of divalent cations. While an excess of Mg is required for the association of rHK to rat mitochondria, the dissociation of bound hexokinase from either rat brain or rat liver mitochondria is not affected by addition of EDTA in excess, which successfully releases the bound enzyme from yeast mitochondria. As in the binding experiments, G6P by itself is ineffective, and only the combination of EDTA and the hexose-phosphate acting together results in efficient release of rHK from rat mitochondria, independently of the presence of dextran.

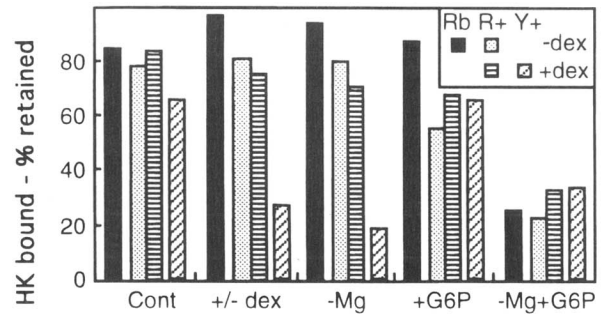

Fig. 6. Effect of dextran, Mg, and G6P on the stability of the complex mitochondria–rHK. Mitochondrial pellets (0.2 mg protein) from each basic binding mix described in Fig. 5 (containing 16–20 mU bound rHK, Cont) were further resuspended in the indicated media, and incubated on ice for one additional hour. In addition, 0.2 mg crude rat brain (Rb) mitochondria pellets containing 10 mU bound rHK was resuspended similarly. The resuspension media (see Fig. 5) contained 25% dextran as indicated, except for the category labeled +/− dex in which the polymer content was opposite to that indicated. The bound rHK fraction in all the resulting mitochondrial pellets was determined to assess the rHK activity retained on mitochondria under the various incubation conditions.

The results indicate that (i) dextran enhances both the binding and the retention of rHK to mitochondria from yeast and, to a lesser extent, from rat; (ii) G6P has no significant influence on the formation or stability of both the rHK:Y+ and rHK:R+ complexes; (iii) Mg^{2+} ions are essential for binding of rHK to both mitochondria; (iv) this requirement is sustained for retention to yeast but not to rat mitochondria.

DISCUSSION

Mitochondrial porin has been proposed to represent the hexokinase receptor, based on a considerable body of experimental indications (Fiek *et al.*, 1982; Kottke *et al.*, 1988; Nakashima *et al.*, 1986; Wilson, 1995), including the demonstration of specific binding of rHK to asolectin vesicles reconstituted with purified rat porin as the sole protein (Felgner *et al.*, 1979).

The present results clearly indicate specific and saturatable binding of rat brain hexokinase to yeast mitochondria containing porin (Yh′ and Y+), with variable apparent affinity and extent, both lower than those assessed for rat mitochondria (Figs. 2–4, Table II). Based on the molecular mass and specific activity for rHK (55 U/mg), together with the porin content of R+ and Y+ mitochondria, molar rHK:porin respective stoichiometries of 1.7 and 1.4 were derived for maximal binding of hexokinase (in the presence of dextran, Fig. 3B). These values could be underestimated since rHK may preferentially bind to a special pool of porin enriched in contact sites (Dorbani *et al.*, 1987; Kabir and Wilson, 1993; Wicker *et al.*, 1993). Assuming rHK is bound as a tetramer, the stoichiometry suggests that porin occurs as a dimer in the binding site.

Despite the low sequence homology between mammalian and yeast porin (Benz, 1994; Blachly-Dyson *et al.*, 1993; Forte *et al.*, 1987), some recognition of rHK still occurs, in correlation with the evolutionary distance from rat to the organisms contributing the porin. With R+, Yh′, and Y+, the extent and apparent affinity for binding (Table II) are both enhanced in the presence of dextran. The results obtained with human porin may be underestimated since the recombinant gene (HVDAC1-HA) product, while functional and properly localized in yeast mito-

chondria (Yu *et al.*, 1995), is slightly different from the original at its C-terminal (10 codons of the hemagglutinin tag added). The binding of rHK to yeast mitochondria expressing other recombinant (including human) porins is under current investigation in greater detail.

On the other hand, porinless mitochondria (Y−) bind rHK without reaching saturation after exceeding more than three times the binding capacity of mitochondria from the same strain, but expressing human porin (Yh′, see Fig. 3B). However, no such "nonspecific" binding is observed with porin-containing mitochondria, independently of the source of mitochondria or porin, even in a dextran-crowded environment which enhances macromolecular interactions. Finally, porinless mitochondria are characterized by a much lower apparent affinity for hexokinase, obvious in the absence of dextran (see Fig. 2A).

The porinless yeast mutants remaining viable on nonfermentative carbon sources (Blachly-Dyson *et al.*, 1990), they may have developed, or rather overexpressed, alternative mitochondrial pores fulfilling the role of porin (Benz *et al.*, 1989; Dihanich *et al.*, 1987). These putative pores may also bind hexokinase in a different configuration. A different porin-like protein, recently discovered and partially characterized by Forte's group as a nonfunctional channel, does not appear to represent a good candidate for the low-affinity/high-capacity hexokinase binding factor in Y− since it is expressed at a similar level in both Y+ and Y− mitochondria (Blachly-Dyson *et al.*, 1997). An alternative simple explanation for the experimental facts is that the native hexokinase binding site includes—but is not restricted to—porin. The outer membrane pore may indeed occur as a membrane protein complex (Dorbani *et al.*, 1987; Krause *et al.*, 1986; Nakashima *et al.*, 1986). In mitochondria from the porinless yeast mutant, latent binding sites contributed by other membrane proteins normally associated with porin may become exposed upon—or within—the outer membrane, and made available for interaction with hexokinase. Accordingly, one may speculate that in the native hexokinase binding site, one molecule of the enzyme may interact with membranal components simultaneously at molecular interfaces in a large complex organized around porin. In the absence of porin, the complex may fail to assemble in the outer membrane, but each of the other components retains some ability for hexokinase binding. In any case, the present study supports a major but not exclusive (see also Nakashima *et al.*, 1986) role for porin in hexokinase binding.

The association of brain hexokinase to porinless yeast mitochondria raises the question whether the mode of binding to yeast mitochondria (wild type or recombinants, expressing heterologous porin) is functionally competent in channeling nucleotides, as it operates in the homologous system. The mode of association of rHK to different mitochondria was indirectly probed using two approaches: (i) examination of the effect of external factors (Mg^{2+}, G6P, and dextran) on the binding, and (ii) analysis of the apparent cooperativity in binding.

Binding of rHK to all mitochondria (including those from the porinless mutant, not shown) requires the presence of Mg^{2+}, independently of the presence of dextran or G6P (Fig. 5). In this respect no distinction is apparent between the heterologous and the homologous systems. Preliminary tests indicate slight quantitative differences among the various mitochondria in the requirement for multivalent cations and their relative effectiveness (not shown), but their presence seems mandatory for achieving efficient binding of rHK. It is yet unclear whether they fulfill a need to reduce a delocalized negative surface charge density (to enable the approach of rHK from solution), or to neutralize specific negative charges (phospholipids or membrane proteins) localized at the binding site, creating a stabilizing electrostatic bridge. The latter point, under further investigation, is corroborated by the differential effect of EDTA on the retention of prebound rHK to rat and yeast mitochondria (Fig. 6). The sensitivity to EDTA observed with rHK:Y+ complexes indicates that cations involved in salt bridges (between rHK and membrane components) may be less accessible to the chelator in the case of the homologous system, as it might be expected for a tighter rHK:Mg:R+ complex. It is indeed worthwhile noting that the release of enzyme effected by EDTA is well correlated with that observed upon resuspension of the preformed complexes in the absence of dextran. In the homologous systems, release of the enzyme is effected by a synergistic action of both EDTA and G6P (Felgner and Wilson, 1977; Kabir and Wilson, 1993; Kurokawa *et al.*, 1983; Wilson, 1968). Thus a conformational change in the hexokinase molecule seems to be required to make Mg^{2+} in the salt bridge accessible to chelation, finally leading to rHK release from rat mitochondria. The lack of this requirement with yeast mitochondria may indicate a different conformation

for the bound rHK, which should be reflected in the binding process.

Titration of porin-containing mitochondria with rHK occurs as a biphasic process with respect to the apparent cooperativity (Fig. 4). With all mitochondria, a transition from low to high Hill coefficient is observed at limiting and excess rHK, respectively. The homologous system (rHK:R+) is characterized by a Hill coefficient of 4, concurring with the proposal that rHK may be bound in a tetrameric form (Xie and Wilson, 1990). However, lower apparent cooperativity (n_H = 2.5) is observed for binding to yeast mitochondria (heterologous systems), which occurs at lower apparent affinity, suggesting that the mode of binding may vary with the source of the organelles. Nevertheless, with porin-containing yeast mitochondria in the presence of dextran, both the apparent affinity to rHK and the cooperativity of its binding reach values close to those obtained in the homologous system (Table II). Similar effects for dextran were observed with binding to intact rat mitochondria, but not to reconstituted outer membrane vesicles nor to uncoupled mitochondria (Wicker *et al.*, 1993). Taken together with our results, these facts point toward an important role for a definite structural organization of the mitochondrial components in binding rHK. Indeed, the transition from noncooperative to cooperative binding (curved Hill plots) does not support a static conformation for the mitochondrial binding sites, nor a mechanism involving oligomerization of rHK in solution as a required step before binding can take place. In the latter case one would expect macromolecular crowding to result in an increase of the apparent n_H in the low [rHK] range, rather than the observed decrease. We propose that binding of the first rHK molecule effects a chain of structural changes in which (i) the formation of a complex between mitochondrial membrane proteins is initiated; (ii) the resulting complexes bind cooperatively additional rHK molecules. The structural changes may also involve the fusion between the inner and outer membranes, as well as rearrangement of lipids (cardiolipin and cholesterol) around the binding site. The latter considerations represent possible sources for the subtle difference in binding rHK to yeast vs. rat mitochondria.

Diffusional restrictions on nucleotides have been implicated in many cellular processes (Aflalo, 1991; Aflalo and Shavit, 1983; Aw and Jones, 1985; Saks *et al.*, 1993), among which oxidative phosphorylation in yeast mitochondria coupled to yeast hexokinase in solution has been modeled (Aflalo and Segel, 1992) and experimentally tested (Aflalo, 1997) using recombinant firefly luciferase localized at the outer mitochondrial membrane in yeast (Aflalo, 1990). The study of nucleotides channeling between the matrix and rHK at the outer membrane of yeast mitochondria by an engineered local probe represents a powerful experimental model system to reexamine cellular processes *in situ*.

CONCLUSIONS

We show that rat brain hexokinase binds in significant amounts to yeast mitochondria. The affinity and extent of binding depend on the origin of porin, and both are enhanced in the presence of dextran. Porinless mitochondria also bind hexokinase, but this occurs by a different mechanism. These preliminary data reveal a rather complex nature for the interaction between mammalian brain hexokinase and mitochondria. Many physicochemical factors are involved in the association between mammalian hexokinase and mitochondria, among which porin plays a major but not exclusive role which can be addressed by molecular studies conducted in yeast.

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