Binding of Rat Brain Hexokinase to Recombinant Yeast Mitochondria: Identification of Necessary Molecular Determinants[1]

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The association *in vitro* of rat brain hexokinase to mitochondria from rat liver or yeast (wild type, porinless, or expressing recombinant human porin) was studied in an effort to identify minimal requirements for each component. A short hydrophobic N-terminal peptide of hexokinase, readily cleavable by proteases, is absolutely required for its binding to all mitochondria. Mammalian porins are significantly cleaved at two positions in putative cytoplasmic loops around residues 110 and 200, as determined by proteolytic-fragment identification using antibodies. Recombinant human porin in yeast mitochondria is more sensitive to proteolysis than wild-type porin in rat liver mitochondria. Recombinant yeast mitochondria, harboring several natural or engineered porins from various sources, bind hexokinase to variable extent with marked preference for the mammalian porin1 isoform. Genetic alteration of this isoform at the C-, but not the N-terminal, results in a significant reduction of hexokinase binding ability. Macromolecular crowding (dextran) promotes a stronger association of the enzyme to all recombinant mitochondria, as well as to proteolytically digested organelles. Consequently, brain hexokinase association with heterologous mitochondria (yeast) in these conditions occurs to an extent comparable to that with homologous (rat) mitochondria. The study, also pertinent to the topology and organization of porin in the membrane, represents a necessary first step in the functional investigation of the physiological role of mammalian hexokinase binding to mitochondria in reconstituted heterologous recombinant systems, as models to cellular metabolism.

KEY WORDS: Heterologous expression; mitochondrial porin; VDAC topology; hexokinase binding; macromolecular recognition; cellular organization.

INTRODUCTION

The concept of enzyme ambiquity (Wilson, 1978), a condition in which the cellular location of an enzyme is variable, has been introduced to address a potential regulatory role for structural organization of cellular metabolism. The reversible and glucose 6-phosphate-sensitive binding of specific isoforms of cytoplasmic mammalian hexokinase to the surface of mitochondria in brain (Wilson, 1978), muscle (Aubert-Foucher *et al.*, 1984) or hepatoma cells (Bustamente *et al.*, 1981), represents a classical example for a regulated dynamic rearrangement of catalytic components according to metabolic needs. The mitochondrial receptor for hexokinase has been tentatively identified (Arora and Pedersen, 1988; Blachly-Dyson *et al.*, 1993; Felgner *et al.*, 1979) as porin (or VDAC[2]), the intrinsic outer membrane protein through which nucleotides and other metabolites are transferred between mitochondria and

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the cytosol. As the detailed aspects of this interaction are still a matter of speculation, a general question arises: how has the recognition between a cytosolic enzyme and an organelle evolved at the molecular level? Mammalian hexokinases have been proposed to originate from gene duplication and fusion of an ancestral enzyme; the homologous N-terminal domain of the product has often lost catalytic ability and evolved to confer regulatory and/or intracellular targeting properties (Arora *et al.*, 1993; Wilson, 1995). With the recent determination of the three-dimensional (3D) structure for hexokinase from rat brain (Mulichak *et al.*, 1998) and human brain (Aleshin *et al.*, 1998), detailed spatial information on variable conformations of the enzyme is also available. In comparison, the molecular determinants on porin responsible for the recognition of hexokinase are largely unknown.

The structure of eukaryotic porin has been predicted to be a 12–19 transmembrane β-strand barrel by analogy to that of their prokaryotic homologs, on the basis of hydropathy distribution profiles (Benz, 1994; Blachly-Dyson *et al.*, 1990; De Pinto *et al.*, 1991; Song and Colombini, 1996). A variety of genes encoding for mitochondrial porin are now available (Blachly-Dyson *et al.*, 1990; Elkeles *et al.*, 1997; Sampson *et al.*, 1997). Their products, as well as site-directed mutated versions, have been expressed in yeast porinless mutants (Blachly-Dyson *et al.*, 1990; 1993; 1997), and partially characterized, mainly in terms of their functionality as voltage-dependent channels of the mitochondrial outer membrane.

Recombinant yeast mitochondria harboring various porins retain the basic capacity to bind rat brain-hexokinase, but not yeast hexokinase, to a variable extent. Although lower than that observed with homologous (rat liver) mitochondria, the binding to yeast mitochondria is further enhanced in the presence of dextran (Aflalo and Azoulay, 1998; Wicker *et al.*, 1993) as the result of macromolecular crowding (Minton, 1993). Moreover, the cooperative binding of hexokinase in these heterologous systems is very similar to that observed with rat mitochondria (Wicker *et al.*, 1993; Xie and Wilson, 1990), yet different from that obtained with porin-less yeast mitochondria (Aflalo and Azoulay, 1998). The relatively low binding of hexokinase to porinless mitochondria indicates an important, but not exclusive, role for mitochondrial porin in the association. On the other hand, VDAC has also been proposed to mediate cellular functions that are not necessarily related to its traditional function as a channel in mitochondria (Beutner *et al.*, 1998;

Blachly-Dyson *et al.*, 1997; Yu *et al.*, 1995), or other cellular membranes in vertebrates (Babel *et al.*, 1991; Benz, 1994; Shafir *et al.*, 1998). Unfortunately, the tertiary structure of VDACs has not yet been determined, and its topology in the membrane is still a matter of controversy (Blachly-Dyson *et al.*, 1990; De Pinto *et al.*, 1991; Mannella, 1997). Thus, directed molecular studies of new functions for VDAC are strongly hampered.

The study of rHK binding to mitochondria is amenable to genetic manipulation in yeast and may help to clarify the role of the porin molecule in the association at the molecular level. In a wider perspective, such studies may shed light on its possible involvement in the organization and regulation of other cellular processes. It is legitimate (Wilson, 1997) to address the mode of association of rHK in reconstituted heterologous systems (yeast mitochondria), which may not be identical to that occurring in the homologous systems. The latter has been demonstrated to yield effective channeling of nucleotides between both catalytic systems (Bustamante *et al.*, 1981; Cesar and Wilson, 1998; Laterveer *et al.*, 1995). In the present study, binding of rHK to recombinant yeast mitochondria was investigated in terms of the effect of modification of the enzyme or the organelles by proteolysis, in comparison with the homologous system. Moreover, various porin genes expressed in porinless yeast were screened for their ability to confer a high hexokinase binding ability to mitochondria isolated from the recombinants. The results provide valuable molecular information and may help delimit sites on porin which interact with hexokinase or which fulfill other structural functions in the context of the outer membrane.

EXPERIMENTAL PROCEDURES

Commercially available bovine pancreatic trypsin, porcine chymotrypsin, porcine elastase and *Staphylococcus aureus* V8 protease (Glu-C), were used without further purification. Dextran T-40 (average $M_r = 40,000$), nucleotides, soybean trypsin inhibitor, as well as other protease inhibitors were from commercial sources.

Biological Materials

Adult white rats (var. Sprague-Dawley) were the source of brains and livers for hexokinase (type I, rHK)

and mitochondria (R+) preparations, respectively. *Saccharomyces cerevisiae* strains M3 (Blachly-Dyson *et al.*, 1997), its *por1*⁻ mutant M22-2 (Blachly-Dyson *et al.*, 1990) or its *por2*⁻ mutant M3-2 (Blachly-Dyson *et al.*, 1997), were used for wild-type (Y++) and porinless (Y−+, or Y+−) yeast mitochondria preparations, respectively. The heterologous expression of native porin genes was conducted in the porinless mutant M22-2, under control of the yeast porin1 (YVDAC1) promoter in a low-copy number plasmid (pSEYC58). They include human HVDAC1 or HVDAC2 (Blachly-Dyson *et al.*, 1993); murine MVDAC1, MVDAC2, or MVDAC3 (Sampson *et al.*, 1997); and wheat WVDAC1, WVDAC2, or WVDAC3 (Elkeles *et al.*, 1995). The resulting recombinant strains were denoted Yh1, Yh2, Ym1, Ym2, Ym3, Yw1, Yw2, and Yw3, respectively. A similar expression system for mammalian porins fused to a short hemagglutinin tag (SYPYDVPDYA) at the C-terminus was used for the engineered human porin HVDAC1-HA (Yu *et al.*, 1995), and three engineered murine porins isoforms (W.J. Craigen, unpublished constructs) MVDAC1-HA, MVDAC2-HA, or MVDAC3-HA, yielding the recombinant strains respectively denoted as Yh1′, Ym1′, Ym2′, or Ym3′. Finally, an engineered version of human VDAC1 with 10 N-terminal residues replaced by a homologous YVDAC1 9 residues fragment (Blachly-Dyson *et al.*, 1993), as well as the native gene for *Drosophila melanogaster* porin DMVDAC integrated to the genome of the porinless mutant M22-2 (Blachly-Dyson *et al.*, 1997), were also used for their heterologous expression in yeast; the strains were denoted Yy:h and Ydm, respectively.

Purified mitochondria, isolated from rat liver (Laterveer *et al.*, 1995) and yeast (Aflalo and Azoulay, 1998) according to published procedures, were resuspended at 20 mg/ml in isosmotic medium (0.3 or 0.6 M sorbitol, respectively) containing 1 mg/ml BSA and stored at −70°C. Bindable rat brain hexokinase from isolated brain particulate fraction was purified by affinity chromatography (Aflalo and Azoulay, 1998).

Binding of Rat Brain Hexokinase to Mitochondria

Yeast or rat liver mitochondria (2 mg/ml) were incubated on ice for 1 h with limiting rat brain hexokinase (200–300 mU/ml) in 0.1 ml of isosmotic medium supplemented with Mg-HEPES 5 mM, pH 7.8 and BSA 1 mg/ml. Dextran (25%. w/v) was routinely added

to yeast mitochondria incubations to enhance rHK binding (Aflalo and Azoulay, 1998). Soluble and mitochondria-bound hexokinase fractions were separated and analyzed for free and bound hexokinase activity, respectively, as described (Aflalo and Azoulay, 1998). Hexokinase binding is calculated as the percentage activity in the bound fraction (routinely 70–85%), relative to the sum of the activities found in the bound and free fractions.

Proteolytic Treatment of Hexokinase and Mitochondria

Hexokinase was incubated on ice with proteases at low concentrations (1:25 or 1:100 by weight) in 10 mM glucose, 5 mM Mg-HEPES, and 20 mM K-HEPES, pH 7.8. The treatment was stopped by the addition of an appropriate inhibitor (see legends to figures). Aliquots from the treated hexokinase were kept on ice until analyzed for activity, binding experiments, and Western blot analysis (see below).

Mitochondria were incubated at room temperature in isosmotic solution supplemented with 5 mM Mg-HEPES and 20 mM K-HEPES, pH 7.8, with a high concentration of protease (1:5 by weight). Proteolysis was stopped by the addition of an appropriate inhibitor. In view of the harsh treatment, the mitochondria were further washed twice in isosmotic medium containing 1 mg/ml BSA. Aliquots from the treated mitochondria were taken for binding experiments and Western blot analysis.

In treated hexokinase or mitochondria samples, zero time controls (protease added after the inhibitor) yielded hexokinase-binding values similar to untreated ones.

SDS-PAGE and Western Blot Analysis

Hexokinase samples (2 μg protein) were fractionated by SDS-PAGE on minigels (10% polyacrylamide) according to Laemmli (1970), and the separated proteins were transferred to nitrocellulose (Towbin *et al.*, 1989). Immunodetection with rabbit polyclonal anti-rHK antibodies, or a mouse monoclonal antibody (4D4) specific to a short rHK N-terminal peptide, was done according to a published procedure (Wilson and Smith, 1985), using the appropriate secondary antibody conjugated to alkaline phosphatase. The immu-

noreactive bands were stained using the NBT/BCIP system.

Mitochondrial samples (100 μg protein) were similarly fractionated on 12.5% polyacrylamide minigels and processed as above for Western blot analysis using rabbit polyclonal anti-yeast porin (a gift from G. Schatz), as well as commercial mouse monoclonal anti-human VDAC1 (N-terminal specific, Calbiochem) or mouse monoclonal antihemagglutinin tag (nonapeptide YPYDVPDYA, Boehringer), as primary antibodies.

RESULTS

The binding of rat brain hexokinase to rat mitochondria requires a short hydrophobic N-terminal segment of hexokinase (Polakis and Wilson, 1985), interacting with porin and/or outer membrane components while inserted in the lipid phase (Xie and Wilson, 1988). Our initial studies indicated that the mode of binding to yeast and rat mitochondria are qualitatively similar (Aflalo and Azoulay, 1998). In order to validate this proposal, the requirements above were critically investigated for rHK binding to yeast organelles.

Effect of Proteolytic Digestion of rHK on its Binding Ability

The results for the time course of mild chymotrypsin and trypsin treatments of purified rHK are presented in Fig. 1. After up to 1 hour, the catalytic activity of the enzyme was not affected by the treatments and most (ca. 90%) of the treated rHK migrated as a single band with an apparent M_r of 100,000 on SDS-PAGE, as confirmed by immunodetection in Western blots using a polyclonal antibody against rHK (inset in Fig. 1a). However, when separate, but identical, blots were overlaid with the 4D4 monoclonal antibody, which specifically recognizes an N-terminal epitope (Polakis and Wilson, 1985; Wilson and Smith, 1985), the 100 kDa rHK band gradually lost its intensity (insets in Fig. 1). Since no 4D4-reactive band was detected at lower apparent M_r, the N-terminal epitope must be lost during the PAGE-blotting procedure. Furthermore, when the treated enzyme was tested for its ability to bind to mitochondria, a digestion time-dependent decrease is observed with rat liver (R+), in agreement with previous results (Laterveer et al., 1995; Polakis and Wilson, 1985; Wilson and Smith, 1985), as well
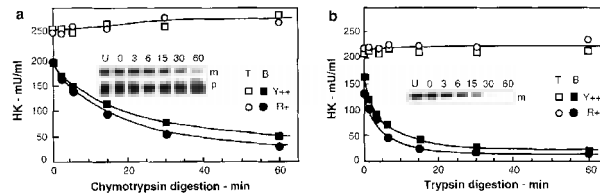

Fig. 1. Effect of mild proteolytic treatment of rHK on its binding to mitochondria. Rat brain hexokinase (0.6 mg/ml) was incubated on ice with (a) chymotrypsin (6 μg/ml) or (b) trypsin (24 μg/ml) for the indicated times, and the reaction was terminated with addition of PMSF (0.5 mM) or trypsin inhibitor (0.48 mg/ml), respectively. Aliquots from the final mix were incubated with R+ or Y++ mitochondria and the hexokinase activity in the mitochondrial (bound, B) and total (bound + free, T) fractions was determined as described in Experimental Procedures. Incubation mixes with yeast mitochondria included 25% dextran. Insets in (a) and (b), Western blot analyses of untreated (U) or parallel aliquots (0.6 μg hexokinase/lane) from the proteolysis time course (0–60 min). The blots were treated with antihexokinase monoclonal (m) and/or polyclonal (p) antibodies, and the bands resolved at an apparent M_r of 100,000 are shown.

as with wild-type (Y++) and porinless (Y−+) yeast (not shown). Quantitative densitometry of the monoclonal antibody-reactive bands intensities along the time course indicate a strong correlation with the reduction in binding ability of both the chymotrypsin and trypsin proteolytic products, in the heterologous (Y++) as well as in the homologous (R+) system (not shown).

This approach was extended to other proteases with different specificity (Fig. 2a). As with trypsin or chymotrypsin, a mild treatment with elastase efficiently removes the N-terminal tail of rHK, as assessed by the reactivity of the 100 kDa band to the 4D4 monoclonal antibody. However, this is observed to a lesser extent with V8 protease, despite a comparable distribution of putative proteolytic sites near the N-terminus. The binding ability of the proteolytic products of rHK to R+ and Y++ as well as to recombinant yeast mitochondria harboring human porin (Yh1′) was assessed. As a control, porinless yeast mitochondria (Y−+), which bind hexokinase less efficiently in an apparently nonsaturable way (Aflalo and Azoulay, 1998), were also tested. The results presented in Fig. 2b confirm that mild proteolytic treatments of purified rHK yield products that have selectively lost a short N-terminal peptide while retaining full catalytic activity. This peptide is essential for binding to rat and yeast mitochondria, independent of the presence or the source of porin.
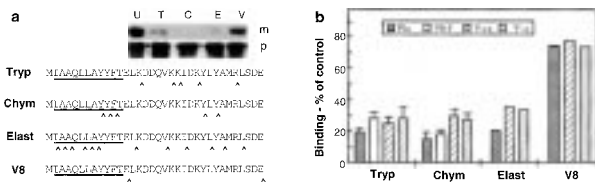
Fig. 2. Differential effect of mild proteolysis of rHK on its binding to different mitochondria. (a) Putative proteolytic sites in the N-terminal 34 residues sequence of rHK. The epitope recognized by the monoclonal antibody 4D4 is underlined. Inset, Western blot analysis of rHK, untreated (U) or digested with trypsin (Tryp, T), chymotrypsin (Chym, C), elastase (Elast, E) or V8 protease (V8, V), as described in Fig. 1. (b) Binding of treated rHK to the indicated mitochondria. Hexokinase (0.6 mg/ml) was incubated on ice for 1 h with trypsin, chymotrypsin, elastase, or V8 protease at a 1:100 ratio (by weight). The digestion was stopped by addition of trypsin inhibitor (0.12 mg/ml), PMSF (0.5 m*M*), elastatinal (0.12 mg/ml), or PMSF (0.5 m*M*), respectively. Aliquots were processed as described in Fig. 1. In all cases, the total catalytic ability of hexokinase was not affected by the treatment. The binding results are expressed relatively to these obtained with untreated rHK controls. The average values for untreated rHK binding by R+, Yh1′ Y++ and Y−+ mitochondria were 72, 72, 71, and 65% of the total rHK added to the incubation, respectively. The averages and standard errors of two experiments are given for the treatments with trypsin and chymotrypsin.

Effect of Proteolytic Digestion of Mitochondria on rHK Binding Ability

In order to affect the mitochondrial receptor(s) for rHK, the reciprocal experiment was performed in which rat and yeast mitochondria containing different porins were subjected to proteolysis and further tested for the binding of native rHK. In view of the high resistance of the outer membrane porin to proteases in intact mitochondria (De Pinto *et al.*, 1991; Felgner *et al.*, 1979; Mihara *et al.*, 1982), a harsh treatment of the organelles was performed; since rHK is sensitive to proteolysis, particular care was taken to inactivate and wash out excess protease before the binding assay.

The results of rHK binding to treated mitochondria indicate differential sensitivities of the tested mitochondria to proteases (Fig. 3). The binding of rHK to rat liver (R+) or wild-type yeast (Y++) mitochondria is essentially resistant to the trypsin treatment. However, while binding to human porin-containing yeast mitochondria (Yh1′) is slightly more sensitive, trypsin digestion of porinless yeast mitochondria (Y−+) reduced their binding capacity by half. A severe treatment with chymotrypsin does not affect the binding ability of rat and yeast mitochondria, independent of the presence or the source of porin. The treatment
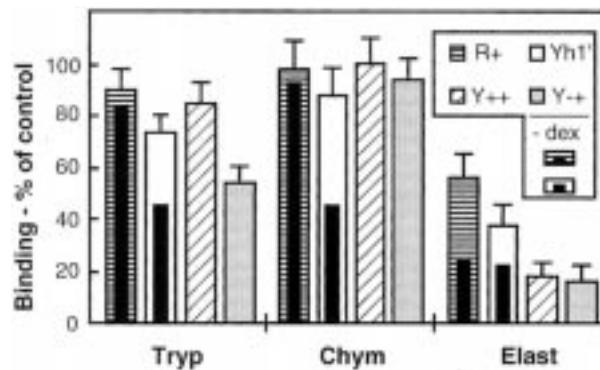

Fig. 3. Differential effect of harsh proteolytic treatments of mitochondria on their ability to bind rat brain hexokinase. The indicated mitochondria (1.2 mg/ml) were incubated at room temperature for 1 h with a high concentration (0.24 mg/ml) of trypsin (Tryp), chymotrypsin (Chym), or elastase (Elast). The reactions were stopped by adding trypsin inhibitor (4.8 mg/ml), PMSF (1 m*M*) or elastatinal (4.8 mg/ml), respectively. Washed aliquots of the mitochondria (0.2 mg protein) were used for rHK (25 mU) binding experiments in the presence of 25% dextran. The results are expressed as in Fig. 2b and represent averages and standard errors of two to three experiments. The average values for untreated rHK binding by R+, Yh1′, Y++, and Y−+ mitochondria were 77, 78, 76, and 62% of the total rHK added to the incubation, respectively. A parallel binding experiment was performed once in the absence of dextran with treated R+ and Yh1′ (black bars). In this condition, the extent of rHK binding to untreated Yh1′ was reduced by a factor of 2.5, while it remained essentially constant with R+.

of mitochondria with elastase is the most effective, significantly affecting binding to R+ or Yh1′, while abolishing completely the binding ability of Y++ and Y−+ mitochondria.

Although we routinely include 25% dextran in our yeast mitochondria binding assays, the association of rHK to Yh1′ in dextran-free media is substantial (Blachly-Dyson *et al.*, 1993) and comparable to the homologous system (Aflalo and Azoulay, 1998). The inhibition of binding to protease-treated mitochondria is stronger in the absence of dextran (narrow black bars in Fig. 3). This result, demonstrating a higher stringency for the assessment of the effect of proteolysis, indicates a possible stabilizing effect of dextran on the organization of the membrane. It should be noted that the inhibition of mitochondrial binding ability we observe may underestimate the true effect, since a small excess of mitochondria over hexokinase is routinely used in our standard assay.

Western blots of the treated mitochondria are presented in Fig. 4 and the results of their qualitative analysis summarized in Table I. For visualization, schematic representations of the porin molecule are
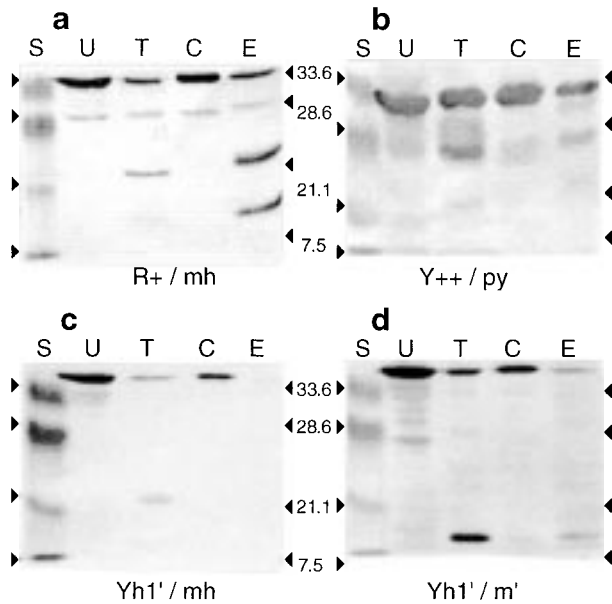
Fig. 4. Western blot analysis of porin fragments in proteolytically treated mitochondria. The indicated mitochondria were treated with trypsin (T), chymotrypsin (C), or elastase (E) as described in Fig. 3. Aliquots (100 μg) from untreated (U) or digested and washed mitochondria were run on SDS-PAGE along with prestained molecular mass standards (S) and processed for Western blotting as described in Experimental Procedures. In these conditions, the lower limit for the size of detectable and resolvable bands is estimated to be 5 kDa. (a) R+ mitochondria products detected with mouse monoclonal antihuman VDAC1, N-terminal specific (mh); (b) Y++ products detected with rabbit polyclonal antiyeast porin (py); (c and d) identical aliquots of recombinant Yh1′ mitochondria products detected with mouse monoclonal antibody against either human VDAC1 (mh) or the hemagglutinin tag (m′), respectively.

presented in Fig. 5, including partial alignments of predicted loops in eukaryotic porins in two debated models for their topology (Benz, 1994; Song and Colombini, 1996). Porin in R+ and Yh1′ were detected as two isoforms or a single band, respectively. Beside the minor (faster) isoform in rat, their apparent M_r's were slightly higher than expected from their length (283 or 293 residues, respectively), in agreement with previous reports (Blachly-Dyson *et al.*, 1993; De Pinto *et al.*, 1991), and in contrast with yeast porin, which migrated normally (Fig. 4b). The minor isoform in rat was resistant to cleavage in our conditions. The results for yeast porin are ambiguous because of significant cross reactivity of the polyclonal antibody with other proteins, also apparent in samples from porin-less mitochondria (not shown).

Mammalian porins (in R+ and Yh1′) are substantially cleaved by trypsin into at least two major polypeptides, one of which (23 kDa)—detected in both isoforms—includes the N-terminus (Fig. 4a,c), and the other (12 kDa)—apparent in Yh1′—contains the C-terminus. The original porin sequence in the latter is, in fact, smaller by about 1 kDa (hemagglutinin tag in Yh1′). The combined results suggest that both mammalian porins are cleaved by trypsin in a loop at residues K200/K201 (see Fig. 5). The distribution of the signal intensity (see Table I) indicates the existence of additional sites exposed to trypsin close to the N-terminal in recombinant human porin (Yh1′) and, to a lesser extent, in rat porin (R+). Wild-type yeast porin appears to be weakly cut by trypsin, yielding a band

Table I. Identification of Major Proteolytic Products of Porin in Treated Mitochondria[a]

Mitochondria (antibody)	Native		Trypsin		Chymotrypsin		Elastase	
	$M_r \times 10^{-3}$	ρ (%)	$M_r \times 10^{-3}$	ρ (%)	$M_r \times 10^{-3}$	ρ (%)	$M_r \times 10^{-3}$	ρ (%)
R+ (mh)	34	(100)	34	(40)	34	(120)	34	(40)
	30[b]	(20)	23	(30)			23	(35)
							12	(35)
Yh1′ (mh)	35	(100)	35	(20)	35	(40)	None	
	32–33	(10)	23	(10)				
Yh1′ (m′)	35	(100)	35	(35)	35	(70)	35	(10)
	28–34	(20)	12	(60)			12	(20)
Y++ (py)	31	(100)	31	(90)	31	(90)	31	(50)
	10–120[c]	(60)						
Y−+ (py)	10–120[c]	(80)			Degradation			

[a] The electrophoretic bands in Fig. 4 were analyzed for apparent size (M_r) by interpolation in molecular weight standard curves. The signal intensity (ρ) was assessed by densitometry of the digitized images and is expressed as the percentage of that found in the main porin band in untreated samples. The different antibodies are denoted as in Fig. 4.

[b] Rat porin isoform, unaffected by proteolysis.

[c] Nonspecific bands due to cross reactivity with the polyclonal antibody.
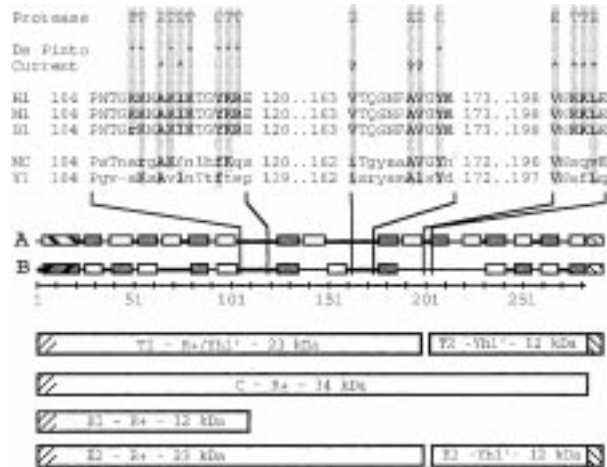
Fig. 5. Accessible proteolytic sites on eukaryotic porins in intact mitochondria. Upper panel: partial alignment of mammalian (H1, human, Swiss-Prot # P21796; M1, murine, GenBank # U30840; B1, bovine, Swiss-Prot # P45879) and fungi (NC, *N. crassa*, Swiss-Prot # P07144; Y1, *S. cerevisiae*, Swiss-Prot # P048040) VDAC1 sequences belonging to three putative loops. Identical residues are in upper case, with homologous potential proteolytic sites for trypsin (T), chymotrypsin (C), and elastase (E) indicated in bold. Conserved proteolytic sites, some of them identified (De Pinto *et al.*, 1991) in mammalian mitochondria by De Pinto (*), or in the current work (^) are highlighted. In the middle panel, two diverging current models for VDAC topology are presented. Boxes represent predicted transmembrane inward (shaded) or outward (open) β-strands (smooth), or a consensus amphipathic α-helix (hatched) exposed on the cytoplasmic interface in model A (Benz, 1994; De Pinto *et al.*, 1991), or crossing the membrane inward in model B (Blachly-Dyson *et al.*, 1990; Song and Colombini, 1996). Predicted cytoplasmic and internal aqueous loops are drawn as thick and thin lines, respectively. Finally, the C-terminal tag in Yh1′ (striped box) is cytoplasmic in A and internalized in B. The lower panel represents the proteolytic fragments identified in Fig. 4, and quantified in Table I. The N- and C-terminal epitopes are striped.

of apparent M_r 27,000 (Fig. 4b), whose identity as a degradation product of porin or another cross-reactive protein remains uncertain.

A similar treatment with chymotrypsin did not affect porin in the wild-type systems (R+ and Y++). In the treated recombinant Yh1′, the signals in both the N-and the C-terminals are significantly reduced with no appearance of lower M_r bands, indicating that only cleavage of short (< 5 kDa) peptides at the extremities of the recombinant porin may have occurred.

In contrast, elastase efficiently cleaved the three porins in R+, Yh1′, and Y++. Rat porin in R+ is substantially cut (see Table I), generating two distinct fragments, which contain the N-terminal, corresponding to likely cleavage sites at V198/L202 and A111/

I113, respectively (see Fig. 5). Tagged human porin in Yh1′ seems to be completely digested, yielding no large final products containing the N-terminal (Fig. 4c), and only a faint band of 12 kDa (Fig. 4d) containing the C-terminal tag. Thus, the recombinant human porin in yeast appears to become destabilized in the membrane during the treatment, in contrast with the effect of trypsin. The results with wild-type yeast porin indicate only a moderate extent for the cleavage by elastase (Fig. 4b).

When the results of proteolysis are compared with these for binding ability (Fig. 3), it appears that the extent of porin cleavage by proteases is not well correlated with the inhibition of rHK binding, especially in the presence of dextran. In addition, since significant binding of hexokinase occurs to porinless mitochondria under molecular crowding conditions (Figs. 2 and 3), these conditions seem inadequate for critically inferring a role for porin in the binding. A different approach was used in which the requirement for porin in the membranes has been investigated more stringently and in a wider range.

Specific Contribution of Porin to High Hexokinase Binding Ability by Yeast Mitochondria

Thus far, the hexokinase receptor on the more complex mitochondrial side is still poorly defined in terms of its molecular components. The net contribution of porin in hexokinase binding was directly evaluated in a screening experiment using yeast mitochondria harboring different porins.

Hexokinase-binding assays to yeast mitochondria harboring 14 natural or engineered porin isoforms (from animal or plant origin) were performed both in the presence and in the absence of dextran. The analysis included, in addition, the results for rat liver mitochondria (homologous system as a positive control), as well as for yeast mitochondria isolated from wild-type (Y++) or two mutated strains $por1^-$ (Y−+) or $por2^-$ (Y+−), devoid of either (1) the functional yeast porin (Blachly-Dyson *et al.*, 1990) or (2) a recently characterized porinlike isoform, which is not functional as a pore (Blachly-Dyson *et al.*, 1997), respectively. A control consisting of the porin-less strain Y−+ expressing the wild-type yeast porin (YVDAC1) gene on a plasmid behaved identically as the wild-type parental strain Y++ (not shown). The host strain Y−+ yields mitochondria, which bind hexokinase

weakly (Aflalo and Azoulay, 1998), and can be considered as a negative control in our screening procedure. The results summarized in Fig. 6 show that in the presence of dextran, a high hexokinase binding ability is shared by all mitochondria tested. However, in the absence of the macromolecular crowding agent, a variable degree of binding is observed. The binding assays, performed using a relative excess of mitochondria over rHK (at limiting concentration), are reproducible (3–12 experiments with each strain) and consistent in relation to the effect of dextran. In the absence of dextran, the extent of binding (as percentage of total rHK) reflects the affinity of rHK to the mitochondria. Thus the different gene products for porin in yeast mitochondria can be classified into three major groups with respect to rHK binding: (1) native mammalian porin 1 isoforms (Yh1, Ym1) and an engineered version of human porin1, in which a short N-terminal fragment was replaced by the homologous yeast sequence (Yy:h), bind hexokinase with a high affinity, comparable to that assessed in the homologous system (R+); (2) other native isoforms from insect (Ydm), mammalian (Yh2, Ym2, Ym3), or plant (Yw1, Yw2, Yw3) origin, as well as mammalian porin1 isoforms tagged at their C-terminus with a short negatively charged hemagglutinin sequence (Yh1′, Ym1′) show a significantly lower affinity for rHK; (3) finally, the worst hexokinase binding group includes tagged mammalian porin2 and porin3 isoforms (Ym2′, Ym3′) and the wild-type yeast porin1 isoform (in Y++ and Y+−). The mitochondria in the latter group bind rHK in the absence of dextran only slightly better than the negative control (Y−+), which itself binds rHK as poorly as the nonbindable yeast hexokinase (Azoulay and Aflalo, 1996), at the

limit of our resolution. They are, nevertheless, distinct from Y−+ since with porin-containing mitochondria in the presence of dextran, rHK binding reaches saturation (about 1–2 U/mg mitochondrial protein, with variable affinity), while with porinless mitochondria the low-affinity binding does not saturate (up to 4 U/mg) (Aflalo and Azoulay, 1998), indicating a "nonspecific" association to porinless (Y−+) mitochondria.

DISCUSSION

The presence of a short lipophilic N-terminal peptide on rHK is absolutely required for its binding (Figs. 1 and 2). This peptide is markedly susceptible to cleavage by chymotrypsin, trypsin, and elastase (but to a lesser extent by V8 protease), as compared to other potential proteolytic sites, which may be less exposed at the surface of the enzyme in aqueous solution. A corresponding gene fragment (encoding for the 15 first residues of rHK), when fused upstream to reporter genes, has been shown to be sufficient to specifically direct the chimeric products to mitochondria, when expressed in mammalian cells (Gelb *et al.*, 1992; Sui and Wilson, 1997). The crystal structure of a dimeric form of rHK indicates that the 15 N-terminal residues may fold into an α-helix, stabilized in the presence of Ca^{2+} by the proximity of a second N-terminal helix from a different dimer (Aleshin *et al.*, 1998; Mulichak *et al.*, 1998). In another similar dimeric crystal form obtained in the absence of Ca^{2+}, the N-terminal 19 residues are disordered and cannot be resolved, indicating that they may assume a loose "random" fold in aqueous solution. Such a conformation should be particularly sensitive to proteolysis. On the other hand, it is likely to fold into a helix in the hydrophobic core of the membrane, where it is inserted upon binding (Xie and Wilson, 1988) and becomes much more resistant to proteolytic degradation (Magnani *et al.*, 1994; Stocchi *et al.*, 1995). Moreover, in view of the predicted amphipathicity for the N-terminal as an α-helix, it may benefit from stabilizing interactions with transmembrane amphipathic segments from one or more mitochondrial proteins and thereby mediate the formation of a large multicomponent complex including porin (Beutner *et al.*, 1998) and a tetrameric form of rHK (Xie and Wilson, 1990). However, the spatial arrangement of rHK bound to the mitochondrion may not be directly inferred from the three-dimensional (3D) structure of the dimer in the crystal forms (Aleshin *et al.*, 1998; Mulichak *et al.*, 1998), since in differ-
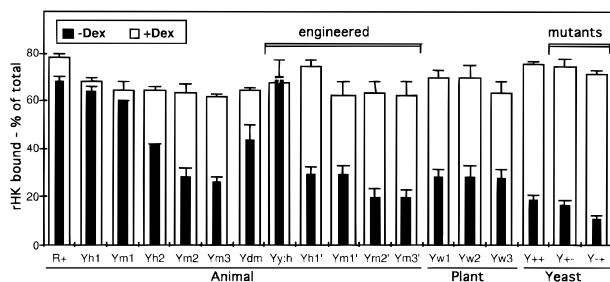

Fig. 6. Differential contribution of recombinant porin in yeast mitochondria to rHK binding. Limiting rHK (0.2–0.3 U/ml) was incubated with excess mitochondria (2 mg/ml) in the absence or presence (25%) of dextran, as indicated, and the samples processed as described in Experimental Procedures. Yeast mitochondria were isolated from the indicated strains, denominated in Experimental Procedures. The results for each strain represent averages and standard errors of 6 to 13 measurements.

ent dimeric crystals the monomers are arranged in a head-to-tail conformation, so that the N-termini are far apart (ca. 13–14 nm), as compared to the estimated outer diameter (ca. 4.8 nm) for a single pore in porin two-dimensional (2D) crystals (Mannella, 1997). Finally, the common requirement of an intact N-terminus in rat brain hexokinase to achieve binding to each of the mitochondria tested is indicative of prevalent properties shared by the organelles. In the presence of dextran, these prevailing—as yet undefined—features are peculiarly independent of the source of the mitochondria, the presence of porin, and often indifferent to their proteolytic treatment. However, this uniform behavior could be observed only under crowded conditions (dextran, see Figs. 3 and 6) and it represents an interesting aspect of functionality under "intracellular" conditions, as discussed further below.

Conversely, evident dissimilarity of the different organelles is demonstrated in the analysis of mitochondrial proteolytic products. It is expected that in conventionally isolated intact mitochondria, only cytoplasmic domains and loops of outer membrane proteins would be exposed to the solution and, therefore, susceptible to exogenous proteases (De Pinto et al., 1991; Mihara et al., 1982). Porin in bovine heart mitochondria has been demonstrated to be cut once by trypsin (between K108 and R119), twice by chymotrypsin (Y117, Y172), and once by V8 protease (D227 or D229) (De Pinto et al., 1991). In our hands, mammalian porin in both rat liver and recombinant yeast mitochondria was cleaved by trypsin in a different loop (see Fig. 5) and no large chymotryptic fragments were found in any mitochondria (Fig. 4), although detergent-solubilized porin becomes very sensitive to these proteases (De Pinto et al., 1991; Shafir et al., 1998). Additional sites for trypsin, as well as elastase, are cleaved close to the N-terminus of human porin in yeast mitochondria (Fig. 4c). Moreover, short C-terminal fragments containing the hemagglutinin tag (in Yh1′) are sensitive to chymotrypsin and elastase (Fig. 4d). The general accessibility of the ends and the loop V198-L202 indicate that they are exposed to the medium, corroborating the 16 transmembrane β-strands model for porin folding and topology and arguing against the concurrent [1 α-helix + 12 β-strands] model (A and B in Fig. 5, respectively). Finally, the appearance of a V8 protease product containing the N-terminus was originally interpreted as a cleavage site at residue D227 or D229 of bovine heart mitochondria (De Pinto et al., 1991) and later claimed to represent a site at E176 (Song and Colombini, 1996). However, since neither interpreta-

tion (176 or 228 residues peptide) matches closely the observed size (22–24 kDa) of the N-terminal fragment (De Pinto et al., 1991), we propose that this V8 cleavage site is at residue E203, in the same loop of mammalian porins shown here to be cut by trypsin and elastase (see Figs. 4 and 5).

The differences observed in porin proteolytic pattern are indicative of variance in the availability of cytoplasmic loops to proteases in different mitochondrial environments. Indeed, assuming a common tertiary structure and topology for mitochondrial porins, homologous proteolytic sites are often conserved in such loops (see Fig. 5), conveying a priori a potential sensitivity to external proteases. Their actual cleavage may however be hampered if the sites interact with other molecular species. Accordingly, the P104-E120 loop in mammalian porin may be accessible to trypsin in bovine heart (De Pinto et al., 1991), but not in rat liver or recombinant yeast mitochondria (Figs. 4 and 5), and vice versa for the V198-E203 loop. This rationale may represent the basis for the reported resistance to trypsin of porin in wild-type yeast mitochondria (Mihara et al., 1982). Similarly, proteolytic sites near the N-terminus can be cleaved in the recombinant yeast mitochondria, resulting in the loss of anti-N-terminal-reactive fragments (Fig. 4c), but not in rat liver mitochondria, in which they may occur as a complex with other rat-specific mitochondrial components. It should be noted that if such complexes represent the rHK binding site, a limited cleavage of porin alone may not necessarily abolish subsequent rHK binding (see Fig. 3). On the other hand, the cleavage of other proteins, which contribute to the rHK receptor complex, may reduce the binding despite the presence of an intact porin, as was found with chymotrypsin-treated recombinant yeast mitochondria (Fig. 4cd) and corroborated by the relatively strong inhibition observed with porin-less mitochondria treated with trypsin and elastase (Fig. 3). In this respect, macromolecular crowding, which promotes interaction among membrane proteins (Aflalo and Azoulay, 1998; Laterveer et al., 1995; Minton, 1993; Wicker et al., 1993), substantially attenuates the inhibition of binding in these cases (Fig. 3). Accordingly, a dynamic complex in the membrane acting as a functional hexokinase binding site, dismantled by proteolysis in the absence of dextran, may be restored and/or stabilized under molecular crowding conditions. While readily conceivable, the participation of other outer mitochondrial membrane proteins besides porin in a supramolecular complex containing

hexokinase (Beutner *et al.*, 1998) still remains to be directly demonstrated.

The difference in apparent affinity of various mitochondria to brain hexokinase is blurred under crowded conditions, which thermodynamically favor the interaction (Fig. 6). However, in the absence of dextran, the results demonstrate a strong dependence on the presence and the source of porin. This provides unequivocal support to a direct involvement of the porin molecule in the specific recognition of brain hexokinase and its association (saturatable) to mitochondria. The highest apparent affinities of yeast mitochondria to rHK are conveyed by mammalian porin1 isoforms (in Yh1 and Ym1). Although the sequence for rat porin1 isoform has not been determined yet, this isoform is expected to be present in rat liver and rat brain mitochondria (homologous systems), since an isoform-specific monoclonal antibody to human porin1 strongly react with these organelles (Fig. 4), as well as with mitochondria isolated from yeast expressing mammalian porin 1 (Yh1, Yh1′, Ym1, Ym1′, not shown). It is pertinent to note that high-affinity binding of rHK to recombinant yeast mitochondria expressing a native mammalian VDAC1 gene (lacking the hemagglutinin tag) either on a plasmid (Fig. 6) or integrated to the genome (Azoulay-Zohar *et al.*, manuscript in preparation), can be achieved in the absence of macromolecules. Thus, one may, in principle, exploit human VDAC1 as a reference for rHK binding (best known binder) and analyze the variation of rHK binding in relation to local divergence between the sequence of the porin variants and that of human VDAC1. The most striking finding is that appending a short hydrophilic tag to the C-terminus of any mammalian porin isoform is consistently detrimental to rHK binding ability (see Fig. 6). This result, indicative of steric hindrance to rHK binding, suggests that the C-terminus of porin, which belongs to a strand embedded in the outer membrane (Blachly-Dyson *et al.*, 1990 De Pinto *et al.*, 1991; Mannella, 1997), is located at the cytoplasmic face (see model A in Fig. 5), close (within 1–2 nm) to the rHK binding site on the porin molecule.

With mammalian VDAC2, rHK binding to recombinant yeast mitochondria (Yh2 and Ym2) is reduced in the absence of dextran, as compared to their homologs in Yh1 and Ym1 (Fig. 6). Mammalian VDAC2 differs from VDAC1 by an additional 11 to 12 residues fragment at the N-terminus, in addition to numerous nonconservative point substitutions scattered along the sequence (Blachly-Dyson *et al.*, 1993; Sampson *et al.*, 1997). However, the rHK binding ability of yeast mitochondria harboring an engineered human porin1 (Yy:h) in which a 9-residue N-terminal peptide from yeast porin1 replaced the original N-terminus, indicates a relatively humble contribution of this part of the molecule to rHK binding. Thus, a more detailed molecular analysis of the divergence in VDAC sequences is needed to delimit potential rHK binding sites.

CONCLUSION

We show that rat brain hexokinase binding to yeast mitochondria does not differ in essence from that in the homologous system. The extent of binding shows a similar dependence on the presence of an N-terminal determinant of hexokinase. The binding to yeast mitochondria, enhanced in the presence of dextran, is constrained by the origin or the integrity of porin. However, the detailed contribution of the porin molecule to the recognition between rHK and the mitochondrial membrane needs yet to be resolved. This work demonstrates that a systematic molecular approach, involving genetic engineering of porin and its heterologous expression in yeast, is now able to address molecular recognition issues at the cellular level, as well as other aspects of the involvement of porin in mitochondrial and cellular regulation.

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