HEXOKINASE BINDING SITES ON MITOCHONDRIAL MEMBRANES*

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Summary - Binding sites for brain hexokinase are found on the outer mitochondrial membrane, but are not present on inner mitochondrial or microsomal membranes.

The major portion of the total hexokinase activity in brain is associated with the mitochondria (1). Approximately half of this activity is readily assayable using untreated mitochondria; adopting the terminology suggested by Katzen et al. (2), we shall refer to this portion as "overt". The remaining activity is in latent form (3,4), becoming apparent only after treatment of the mitochondria by a variety of membrane-disrupting techniques (e.g., osmotic shock or freeze-thaw cycles). Disruption of the membranes is not sufficient, however, to release the latent enzyme in soluble form (4), indicating that it retains an association with one or both of the mitochondrial membranes. We have, therefore, investigated the binding of rat brain hexokinase to mitochondrial membranes.

Experimental Procedures

Mitochondrial Membranes - Livers were obtained from Sprague-Dawley rats, 250-350 g., after fasting overnight. Mitochondria and mitochondrial membranes were prepared according to Sottocasa et al. (5), using the

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"3-layer" gradient[1]. The separated mitochondrial membranes were resuspended in 0.25 M sucrose for use.

Assays - Hexokinase (6), RIDCR[2] and adenylate kinase (5), and protein (7) were determined spectrophotometrically by previously published procedures. Cytochrome c oxidase was assayed by following the decrease in absorbance at 550 mμ after addition of enzyme to a cuvette containing 0.03 μmole reduced cytochrome c in 1.0 ml of 0.1 M potassium phosphate - 0.5 mM EDTA, pH 7.0. For these enzymes, one unit is defined as that causing the conversion of one μmole of substrate per min. Monoamine oxidase was assayed fluorometrically (8); one unit is defined as that causing an increase of one arbitrary fluorescence unit per min under our instrumental conditions.

Preparation of Solubilized Brain Hexokinase - The crude mitochondrial fraction (6) was suspended in 0.25 M sucrose - 1 mM glucose-6-phosphate, incubated 15 min at 25°, and centrifuged at 41,000 × g × 15 min. The supernatant containing the solubilized enzyme was used immediately for the binding experiments.

Hexokinase Binding Procedure - Untreated mitochondria or isolated membranes were incubated for 15 min at 25° with appropriate amounts of the solubilized brain hexokinase in 0.25 M sucrose - 3 mM $MgCl_2$. After centrifugation at 41,000 × g × 15 min, binding was calculated from the decrease in hexokinase activity in the supernatant. Hexokinase binding exhibited the behavior expected for an adsorption process, being initially directly proportional to the amount of added mitochondria or membranes, and asymptotically approaching a maximum with excess membranes. In these experiments, binding was

[1]The 15 sec sonication step was carried out with a Branson S-125 sonifier using the maximum power possible (1.5-3.0 amp) without frothing. This limitation resulted in somewhat less extensive separation of the membrane marker enzymes than observed by some other workers (5,9) but reduced the contamination of the outer membrane fraction with inner membranes, an advantage for our purpose since we desired a reasonably accurate measure of the binding ability of the outer membrane.

[2]Abbreviations used: RIDCR, rotenone insensitive DPNH-cytochrome c reductase; BSA, bovine serum albumin.

always conducted under conditions where it was proportional to added membranes or mitochondria since this implied an excess of bindable enzyme.

Results and Discussion

Hexokinase bound to the intact mitochondria prior to separation of the membranes distributes identically to the RIDCR and monoamine oxidase activities (Table I) considered by many investigators as characteristic markers for the outer mitochondrial membrane (e.g., 5,9). Concentration of these activities in the outer membrane fraction is readily apparent from a comparison of the specific activity values. Thus incubation of intact mitochondria with excess brain hexokinase results in binding to the outer membrane exclusively. This is, of course, not surprising since it seems unlikely that the enzyme could penetrate to binding sites on the inner membranes even if they were present.

When the separated membranes were tested for their hexokinase binding ability, it was found that the outer membrane fraction was ten-fold more effective than the inner membrane fraction when compared on a per mg basis (Table I). Since the marker enzyme distribution indicated appreciable contamination of the inner membrane fraction with the avidly-binding outer membranes, the actual difference must be much greater than ten-fold. Furthermore, if additional binding sites on the inner membranes were rendered accessible by removal of the outer membrane, one might expect a preponderance of the total binding ability (the sum of the newly accessible inner membrane sites plus those on the outer membranes remaining in the fraction) to be found in the inner membrane fraction. In fact, the total binding ability of the outer membrane fraction exceeded that of the inner membrane fraction and was actually somewhat greater than would be expected from the marker enzyme distribution, suggesting that separation of the membranes results in exposure of additional binding sites on the outer membrane. Clearly, significant hexokinase binding ability is a unique property of the outer mitochondrial membrane, leading to the conclusion that both the "overt"

TABLE I. Distribution of Enzymatic Activities, Hexokinase Binding Sites, and Protein Between Inner and Outer Mitochondrial Membrane Fractions[a]

	% in Fraction		Specific Activities ($\frac{\text{units}}{\text{mg protein}}$)		
	Inner Membrane	Outer Membrane	Intact Mitochondria	Inner Membrane	Outer Membrane
Bound Hexokinase[b]	52 ± 3	48 ± 3	0.16 ± 0.08	0.05 ± 0.02	0.36 ± 0.01
RIDCR	52 ± 11	48 ± 11	0.17 ± 0.07	0.07 ± 0.03	0.49 ± 0.16
Monoamine Oxidase	55 ± 15	45 ± 15	0.25 ± 0.02	0.12 ± 0.04	1.00 ± 0.15
Hexokinase Binding Sites[c]	45 ± 10	55 ± 10	---	0.05 ± 0.02	0.50 ± 0.07
Cyt c Oxidase	88 ± 9	12 ± 9	0.41 ± 0.20	0.56 ± 0.36	0.41 ± 0.16
Protein	87 ± 8	13 ± 8	---	---	---

[a] The average values from three to seven experiments are given with standard deviations.

[b] The mitochondria were incubated with excess brain hexokinase prior to separation of the inner and outer membranes.

[c] The total hexokinase binding capacity was determined after isolation of the membrane fractions; hexokinase binding is expressed as units of hexokinase activity bound per mg membrane protein.

and "latent" forms of brain hexokinase must be associated with the outer mitochondrial membrane. This is consistent with the recent report of Craven et al. (9) who found the hexokinase activity concentrated in the outer membrane fraction prepared from brain mitochondria.

Our previous suggestion that exposure of the latent brain hexokinase required disruption of the mitochondrial membranes (4), has recently been questioned by Craven et al. (9) who reported that suspension of the mitochondria in 1% BSA caused exposure of the latent activity without detectable change in the light scattering properties of the particles expected to result from disruption of the membranes. Since the osmotic pressure of 1% BSA is essentially that of pure water, one would indeed expect osmotic lysis of the particles to have occurred. That the mitochondrial membranes are disrupted by this procedure is demonstrated by the results in Table II. Osmotic shock, either by suspension in pure water or in 1% BSA, resulted in exposure of the latent hexokinase, as expected, and the release of adenylate kinase, a marker for the soluble portion of the intramitochondrial space (5). A requirement for membrane disruption in order to expose the latent activity could indicate either that the enzyme occupies binding sites on both the inside and the outside of the outer membrane, or that the activity of a major portion of the enzyme bound to the exterior is somehow masked, becoming exposed only as the result of the extensive disorganization likely to accompany membrane disruption.

The current controversy surrounding the use of monoamine oxidase and RIDCR as markers for the outer mitochondrial membrane (e.g., 5,9,10) seems to revolve about the ambiguity resulting from the apparent location of these activities in both the outer mitochondrial membrane and the microsomes. Rose and Warms (11) have reported, and we have confirmed (unpublished results), the absence of significant hexokinase binding by microsomal membranes. Thus hexokinase binding ability appears to be one property which may be used unequivocally as a marker for the outer mitochondrial

TABLE II. Effect of Osmotic Shock on the Hexokinase and Adenylate Kinase Activities of Brain Mitochondria[a]

	Hexokinase (units)				Adenylate Kinase (units)		
	Soluble	Particulate					
Suspending Medium		Overt	Latent	Total	Soluble	Particulate	Total
0.25 M Sucrose	0.21	1.09	0.82	2.12	1.16	6.64	7.80
Water	0.25	2.26	0.10	2.61	6.12	2.54	8.66
1% BSA	0.10	2.44	<0.05	2.54	6.28	2.86	9.14
0.5% Triton in 0.23 M Sucrose	0.82	1.35	<0.05	2.17	7.44	0.88	8.32

[a]The crude mitochondrial fraction was prepared as described in the text, and portions suspended in the indicated media. After centrifugation at 41,000 X g X 15 min, the supernatants were assayed for "soluble activities". The pellets were resuspended in 0.25 M sucrose and assayed for "overt" hexokinase. After treatment with 0.5% Triton X-100, the particulate fractions were again assayed for hexokinase and for adenylate kinase. The increase in hexokinase activity after addition of Triton is defined as "latent" activity.

membrane. The technique of binding hexokinase to the outer membrane of intact mitochondria before preparation of the submitochondrial fractions, as described above, seems particularly convenient.

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