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A COMPARATIVE STUDY ON THE BINDING OF HEXOKINASE TO THE OUTER MITOCHONDRIAL MEMBRANE AND A HYDROPHOBIC MATRIX

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Abstract

A major portion of rat brain hexokinase (HK) is bound to the outer membrane of mitochondria. Glucose-6-phosphate (G6P) can release the bound enzyme from isolated mitochondria in the same manner and ratio that it releases the particulate form of the enzyme from the whole homogenate of rat brain. Mg^{++} and polyamines rebind the solubilized type I hexokinase to partially depleted and HK-free mitochondria both in the presence and absence of G6P. A hydrophobic matrix, palmityl-substituted Sepharose-4B (Sepharose-lipid) was used as an artificial adsorbent. HK readily binds to this matrix with retention of its catalytic activity. Positive cooperativity was seen both in the binding of HK to mitochondria and Sepharose-lipid. G6P, which has a releasing effect on the mitochondria-bound enzyme, enhanced binding of the enzyme to the hydrophobic matrix. Mg^{++} and spermine had a negative effect on binding and antagonized the enhancing effect of G6P. Chymotrypsin treatment of HK which causes loss of binding to mitochondria also resulted in loss of adsorption to the hydrophobic matrix, thus demonstrating that the "hydrophobic tail" present at its N-terminal end is essential for binding in both cases. In the same manner that Mg^{++} and spermine antagonize the releasing effect of G6P on binding to the mitochondrial membrane, they antagonize the enhancing effect of G6P on adsorption to Sepharose-lipid. From the results presented, it is speculated that G6P, by virtue of its ability to cause conformational changes in the enzyme molecule, makes its hydrophobic tail more available for interaction with palmityl residues of the artificial matrix.

Keywords: Hexokinase type I; Outer mitochondrial membrane; Rat brain; Hydrophobic matrix; Sepharose-lipid

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Introduction

Mammalian tissues contain four types of hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1) designated as hexokinase types I-IV, depending on their electrophoretic mobility. The isoenzymes I-III, which have low Km values for glucose, are present in differing

proportions in various tissues. Type IV hexokinase (or glucokinase) is a high Km isoenzyme which is found exclusively in the liver and pancreas [36,37]. Type I isoenzyme of rat brain, like other mammalian hexokinases, consists of a single polypeptide chain having a molecular weight of approximately 100 kDa [29]. This type of enzyme constitutes the predominant form of the enzyme in the brain, a major portion of which is associated with mitochondria. It is a typical example of an "ambiquitous enzyme" [34] which binds reversibly to the outer mitochondrial membrane. An alteration in distribution of hexokinase between the soluble and mitochondrial fractions of the cell has been suggested as a mechanism for control of glucose metabolism in the brain [37]. Early studies have demonstrated that the binding of HK to mitochondria from rat brain or sarcoma 37 ascites tumor cells was reversed by G6P, and this solubilizing effect was antagonized by Mg^{++} [28]. Mitochondria-bound enzyme is more active than the soluble enzyme since the bound form can utilize the ATP generated in mitochondria more preferentially and is less sensitive to G6P inhibition [14,36].

Binding of HK involves insertion of a hydrophobic N-terminal "tail" containing 11 residues possibly exists in the helical structure, into the hydrophobic core of the outer membrane of mitochondria [39]. Limited digestion of HK with chymotrypsin generates a non-bindable enzyme [25]. A protein responsible for the specific interaction of HK with the outer mitochondrial membrane was isolated by Felgner *et al.* [8] and subsequently found to be identical to the pore-forming protein, porin [9,18]. The hydrophobic N-terminal segment is the portion of the enzyme known to be necessary and sufficient for binding of HK to this receptor [19]. Mg^{++} and polyamines promote the binding of solubilized HK type II to mitochondria and reverse the G6P-induced release of bound enzyme [15].

To further study the hydrophobic interaction between the enzyme and the outer membrane of mitochondria, palmityl-substituted Sepharose-4B was used as a model simulating the membrane [22]. This allowed the exclusive investigation of the hydrophobic interaction of the enzyme with a hydrophobic matrix. In contrast, binding to mitochondria always involves two components, a specific binding to porin and a hydrophobic interaction. The effect of the releasing factor, G6P, and the rebinding factors, Mg^{++} and polyamines, on the adsorption of the enzyme on the hydrophobic adsorbent was investigated. Binding of hexokinase to phenyl-Sepharose has been previously reported [16].

Materials and Methods

All chemicals, Tris, Hepes, Triton X-100, monothioglycerol and Sepharose-4B were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Affi-Gel Blue was purchased from Bio-Rad Laboratories (Richmond, CA). Other chemicals were obtained from Sigma Chemical Co. or Merck (Darmstadt). Palmityl-substituted Sepharose-4B was prepared according to the procedure described previously [20].

Brains were obtained from adult male NMRI rats. Animals were killed by decapitation and the brains were rapidly removed and dropped immediately into liquid air where they were stored until use.

Preparation of Mitochondria

This was carried out essentially as described previously [5,33]. Frozen brains were thawed by immersion into ice-cold medium which contained 0.25 M sucrose, 10 mM Hepes, 10 mM thioglycerol, 0.1 mM EDTA, pH 7.4 (SHTE medium). After thawing, brains were homogenized in 10 vol. of the same medium and the mitochondrial fraction was isolated by differential centrifugation. Rat liver mitochondria were prepared by the method of Rickwood *et al.* [27].

Hexokinase Assay

Hexokinase activity was determined spectrophotometrically using a glucose-6-phosphate dehydrogenase-coupled assay [33] and a Shimadzu UV-160 spectrophotometer.

Enzyme Purification

Rat brain HK was purified by affinity chromatography on Affi-Gel Blue [35]. The Amicon filter that was used in this procedure was YM10 (Amicon Corp. MA) and SDS-PAGE was done based on the method of Laemmli [17].

Solubilization of Mitochondria-Bound Enzyme

Suspended mitochondria in SHTE medium were made 2 mM in G6P and, after incubation for 30 min at 28°C, centrifuged at 40000 × g for 20 min. The supernatant fluid containing the G6P-solubilized HK was carefully decanted. When necessary, G6P was removed by dialysis.

Binding of HK to Mitochondria

G6P-solubilized enzyme was rebound on rat brain mitochondria, which were partially depleted from HK by G6P or rat liver mitochondria, naturally devoid of HK activity. Except where noted in the text, brain mitochondria were incubated for 30 min at 28°C with appropriate amounts of solubilized HK in SHT medium (the same as SHTE but without EDTA) containing 2 mM $MgCl_2$ [12]. For binding to liver mitochondria, the concentration of

$MgCl_2$ was 3 mM. After centrifugation for 10 min in an Eppendorf microfuge, binding was calculated by measuring the HK activity in the pellet and the supernatant fluid. These binding studies where done in polypropylene tubes which had been dipped in 2% bovine serum albumin (BSA) solution and dried before use.

Adsorption of HK on Sepharose-Lipid and Measurement of Enzyme Activity on the Column

Sepharose-lipid was placed in two separate columns to a packed volume of 1 ml. One of these columns was washed and equilibrated with the SHT medium containing G6P (2 mM), and loaded by 0.6 units of HK containing 2 mM G6P. The same amount of the enzyme which was free of G6P was loaded on the other column, washed and equilibrated with G6P-free medium. Both columns were then washed with G6P-free medium and the assay mixture containing ATP was passed through the columns with a flow rate of 0.4 ml/min and fractions (5 ml) were collected. To 0.3 ml of each fraction, NADP+(20 µl of 50 mg/ml in 0.1 M sodium phosphate, pH 7.0) and G6PD (20 µl of 100 U/ml) were added and the absorbance was read at 340 nm. In this manner, G6P concentration reflecting HK activity was determined.

Assay of Adsorbed Enzyme in Sepharose-Lipid Suspension

Three ml of Sepharose-lipid was washed by SHT medium (three washes) and incubated with 2 ml of G6P-solubilized HK containing 0.86 units of the enzyme which was dialysed to remove G6P. The suspension was then centrifuged in Whisperfuge and the pellet washed three times by SHT medium. Adsorption of the enzyme was determined by measuring enzyme activity. In a 3 ml cuvette, Sepharose-lipid suspension was added to HK assay mixture in a total volume of 3 ml. The contents of the cuvette were mixed for one minute using a very small magnetic stirrer followed by rapid centrifugation in a Whisperfuge again for one min. Absorbance was subsequently read at 340 nm. By repeating the above steps a value for OD/min was obtained.

Immobilization of Mitochondria on Fractosil-1000

One ml of packed Fractosil-1000 was placed on a column (0.7 × 10) and equilibrated with SHT medium. Rat brain mitochondrial suspension, corresponding to 2.5 mg protein, with the bound HK activity of 1.0 unit/ml was loaded on the column and washed with 50 ml of the medium. To make sure that mitochondria were adsorbed on to this column, rotenone-insensitive NADH-cytochrome c reductase and monoamine oxidase activities were determined as marker enzymes. This was carried out by the passage of enzyme assay mixtures prepared according to Sottocasa *et al.* [31] and Schnaitman *et al.* [30]. Constant flow rates were obtained using peristaltic pumps. Fractions were collected and their absorbance measured. HK activity on this column was determined in the same manner as that used for the (solubilized) enzyme immobilized on Sepharose-lipid column described above.

Cleavage of the Hydrophobic N-Terminal Segment by Alpha Chymotrypsin

Limited digestion of purified HK I by alpha chymotrypsin was carried out in SHT medium and stopped by the addition of phenylmethylsulfonylflouride (PMSF) according to Kurokawa *et al.* [15].

Results

Initial experiments were carried out to study the releasing effect of G6P using whole homogenate and isolated mitochondrial fractions. As seen in Table I, treatment of the particulate form of HK in whole homogenate of rat brain with G6P resulted in an appreciable solubilization of HK. This solubilizing agent showed similar efficacy with either the whole homogenate or purified mitochondria (Table I). Enzyme solubilization in these studies was done according to Chou and Wilson [5]. These results indicate that when the amount of sample is too small to justify preparation of purified mitochondria, the releasing studies can be done using whole homogenate.

Effectiveness of Mg^{++} on Rebinding of Solubilized Hexokinase on Rat Brain Mitochondria in the Presence and Absence of G6P

Rose and Warms [28] demonstrated that during the incubation of rat brain mitochondria with G6P, the addition of Mg^{++} caused rebinding. For determining the effectiveness of Mg^{++} on the rebinding process in the presence and absence of G6P, various amounts of HK-depleted mitochondria were added to the tubes containing a fixed concentration of HK (1U/ml), and the rebinding effect of Mg^{++} in the presence and absence of G6P was studied. As indicated in Figure 1, rebinding curves were very similar in both the presence and absence of G6P.

Rebinding of Solubilized Enzyme on HK-Free Mitochondria by Polyamines

Polyamines (putrescine, spermidine and spermine) have di, tri or tetra amino groups and at physiological pH values act as polyvalent cations. Kurokawa *et al.* [15] reported that spermine and spermidine markedly promoted the binding of HK type II to mitochondria prepared from Ehrlich-Lettre hyperdiploid ascites tumor (ELD) cells. The present results showed that polyamines can also cause rebinding of HK type I from rat brain to HK-free mitochondria of rat liver (Fig. 2). As indicated, the

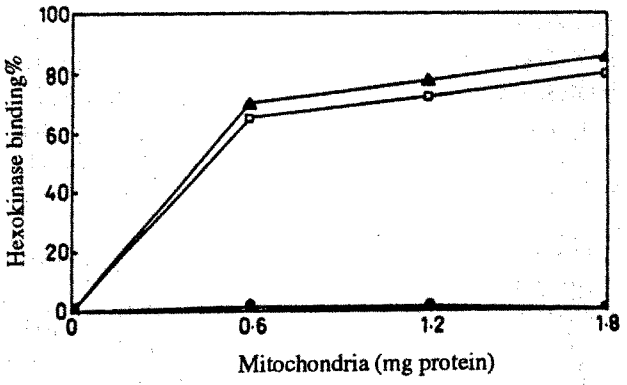

Figure 1. Effectiveness of Mg^{++} on rebinding of solubilized hexokinase to rat brain mitochondria in the presence and absence of G6P. One unit of G6P-solubilized enzyme was incubated for 30 min at 28°C with various amounts of HK-depleted mitochondria in SHT medium (see Materials and Methods) containing 2 mM $MgCl_2$ in the presence (□) and absence (▲) of G6P (2 mM). After centrifugation, percent of bound enzyme was calculated by measuring HK remaining in the supernatant fluid and bound HK in pellet. Control experiments were conducted in the presence (○) and absence (●) of G6P in $MgCl_2$-free medium. In the experiments which were done in the absence of G6P, the G6P was utilized by adding $NADP^+$ and G6PD.

rebinding effect of polyamines, especially that of spermine, was much greater than Mg^{++}.

Effect of G6P on the Adsorption of HK on Sepharose-Lipid

In previous communications [20], we reported on the use of palmityl-substituted Sepharose-4B as a non-ionic matrix for protein adsorption. Such a method of adsorption can be used for immobilization of enzymes with retention of their catalytic activites. As indicated in Figures 3 and 4, rat brain-solubilized HK may be adsorbed on Sepharose-lipid matrix in the form of column or suspension with retention of catalytic activity. G6P, which had a releasing effect on HK bound to the outer surface of mitochondria, markedly enchanced adsorption of HK on Sepharose-lipid particles. To identify the basis of the increased activity on the column, 250 µl of Sepharose-lipid suspension was incubated with an enzyme solution containing 0.55 units of activity in the presence and absence of 2 mM G6P. After centrifugation, the extent of adsorption was determined by measuring the remaining activity of the enzyme in the supernatant fluid. This was found to be 0.054 and 0.135 units in the presence and absence of G6P respectively. Accordingly, it was concluded that the higher observed activity in the column

Table I. Effect of G6P on the release of HK using mitochondrial suspensions or whole homogenates

		HK activity (unit/g brain)			
	Additions	Supernatant	Pellet	Total	Release%
Original Homogenate	None	___	___	11.1	___
(O.H)	Triton	___	___	13.1	___
Centrifugation of O.H	None	2.2	8.9	11.1	___
	Triton	2.9	10.3	13.2	___
Suspension of 105000x g,	None	1.1	6.9	8.0	13.7
pellet	G6P	8.3	3.2	11.5	72.2
	G6P+Triton	11.9	1.8	13.7	86.2
1000 g supernatant	___	2.6	6.4	9.0	___
First wash	___	0.8	6.1	6.9	___
Second wash	___	0.1	5.0	5.1	___
Washed mitochondria	G6P	5.4	1.8	7.2	75
	G6P+Triton	6.9	0.6	7.5	92.7

Original homogenate of rat brains was divided into two fractions. Preparation of mitochondria was done as described in the Materials and Methods section. Releasing procedure was run in two parallel experiments on isolated mitochondria and the original homogenate fraction.

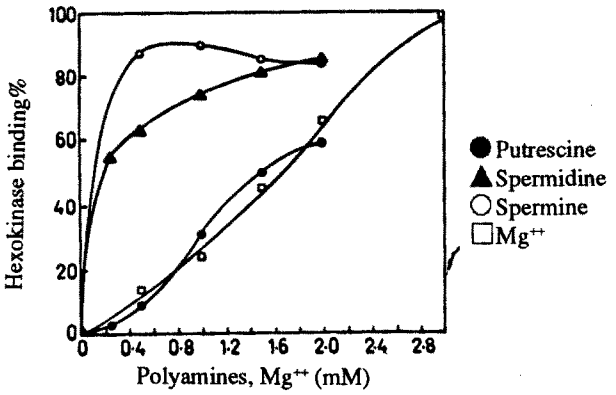

Figure 2. Effect of polyamines and Mg^{++} on rebinding of hexokinase I to rat liver mitochondria. G6P-solubilized hexokinase I (0.16 unit, from rat brain mitochondria), which had been dialysed to remove G6P was incubated with HK-free liver mitochondria (3 mg as protein) in 0.5 ml of 0.25 M sucrose, 10 mM hepes, 10 mM thioglycerol and indicated concentrations of polyamines or Mg^{++}, at pH 7.4 for 30 min at 28°C. After centrifugation, percent of binding was calculated by measuring activity of enzyme in the supernatant fluid and pellet. ●, putrescine; ○, spermine; ▲ spermidine; □ Mg^{++}.

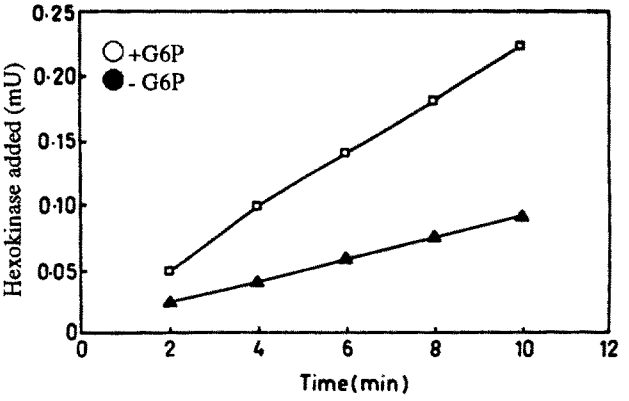

Figure 4. Effect of G6P on the adsorption of hexokinase on Sepharose-lipid suspension. An equal amount (0.86 units) of G6P-free and G6P-containing hexokinase was incubated with the 3 ml of Sepharose-lipid suspension which had been washed and equilibrated with G6P-free and G6P-containing media, respectively. They were then centrifuged and washed three times with G6P-free medium (SHT) and activity of adsorbed enzyme was measured in both conditions, in the presence of G6P (□) and in the absence of G6P (▲).

in the presence of G6P is due to increased adsorption.

Effect of Mg^{++} and Spermine on Adsorption of HK on Sepharose-Lipid

Table II indicates that Mg^{++} and spermine lowered adsorption of HK on Sepharose-lipid particles. When

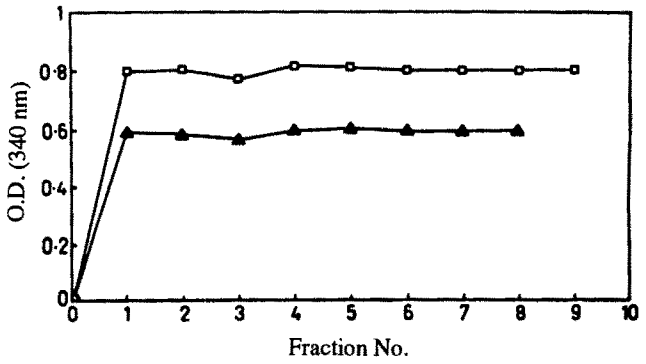

Figure 3. Effect of G6P on the adsorption of hexokinase I on Sepharose-lipid column. Equal amounts (0.6 units) of G6P-free and G6P-containing hexokinase were loaded on two similar Sepharose-lipid columns, washed and equilibrated with G6P-free and G6P-containing (2 mM) SHT medium respectively. Both columns were then washed with G6P-free medium. After passage of assay mixture containing ATP through the columns, fractions were collected. Appropriate amounts of NADP+ and G6PD were added to each fraction and absorbance was read at 340 nm. □ in the presence of G6P, ▲ in the absence of G6P.

Mg^{++} and G6P were added simultaneously to the medium, Mg^{++} antagonized the enchancing effect of G6P on the adsorption of enzyme to Sepharose-lipid matrix.

The Hydrophobic N-Terminal Segment of HK Type I is Essential for Binding of HK to Sepharose-Lipid Matrix and Mitochondria

Upon treatment of the purified rat brain HK with alpha-chymotrypsin, which removes the hydrophobic tail, as a result the binding capacity of HK on enzyme-free rat liver mitochondria (Table III) and Sepharose-lipid is lost (Fig. 5).

Cooperativity in the Binding of HK on Mitochondria and Sepharose-Lipid Matrix

The relationship between the amount of enzyme adsorbed on HK-depleted rat brain mitochondria and the total enzyme concentration carried out in the presence and absence of G6P is depicted in Figure 6. Results obtained using Sepharose-lipid as the adsorbent are shown in Figure 7. As can be seen, adsorption of increasing amounts of HK to a fixed amount of mitochondria and Sepharose-lipid suspension are represented by curves indicating saturation of binding sites which are similar to results presented on titration of rat liver mitochondria with the enzyme [40]. Hill plots of the binding of HK to mitochondria and adsorption on Sepharose-lipid matrix showed positive cooperative interactions at high concentrations of HK observed both in the presence and absence of G6P (Figs.

8 and 9).

Continuous Catalytic Operation Involving HK Bound to Mitochondria or Sepharose-Lipid

Figure 10 shows the use of HK immobilized on Sepharose-lipid matrix in a continuous catalytic process

Table II. Effect of polyamines and Mg^{++} on adsorption of HK on Sepharose-lipid

Additions	Remaining activity (unit) of HK in the supernatant
None	0.36
G6P (2 mM)	0.20
Mg^{++} (2 mM)	0.46
Spermine (0.5 mM)	0.40
G6P (2 mM)+Mg^{++}(2 mM)	0.34

Sepharose-lipid suspension (200 ul) was equilibrated with SHT medium containing indicated additives in separate tubes. To each tube the same amount of enzyme (0.8 units in SHT medium which contained indicated additives) was added and incubated for 10 min at room temperature. After centrifugation, the remaining enzyme activity was measured in the supernatant fluid.

Table III. The role of the N-terminal hydrophobic segment on binding of HK to mitochondria

	HK activity (unit)	
	Supernatant	Pellet
HK+Chym.+PMSF	2.29	0.00
HK+PMSF+Chym.	1.44	0.25
HK+PMSF	2.05	0.29

This experiment was carried out parallel to the experiment depicted in Figure 5. Rat liver mitochondria (0.3 mg as protein) were incubated with chymotrypsin-treated purified hexokinase (2.3 units) for 30 min at 30°C in SHT medium containing Mg^{++} at a final concentration of 3 mM. After centrifugation at 13500 rpm, the activity of HK was measured in the supernatant and resuspended pellet.

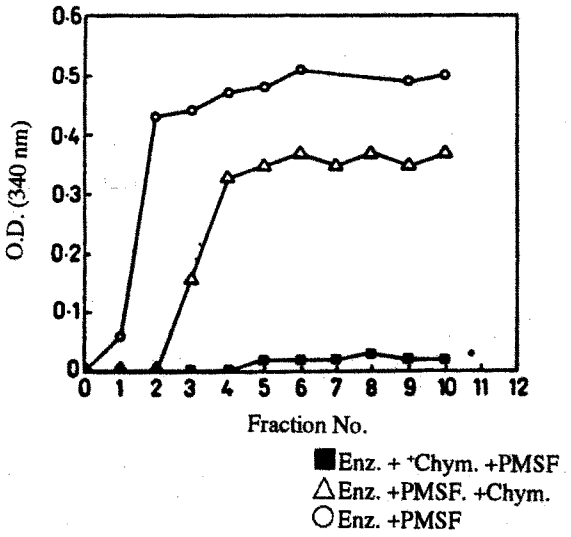

Figure 5. Role of the N-terminal hydrophobic segment of hexokinase on the adsorption on Sepharose-lipid. Purified HK (1.85 U/ml) was treated with α-chymotrypsin (2 μg/ml) and incubated in the SHT medium for 20 min. at 30°C. The reaction was stopped by the addition of PMSF, inhibitor of chymotrypsin (1mM at final concentration), at the end of the incubation period. Then the binding ability of chymotrypsin-treated HK was determined on Sepharose-lipid and liver mitochondria. In control experiments, PMSF was added before chymotrypsin (Δ) or chymotrypsin was omitted and the enzyme was incubated only with PMSF (○).

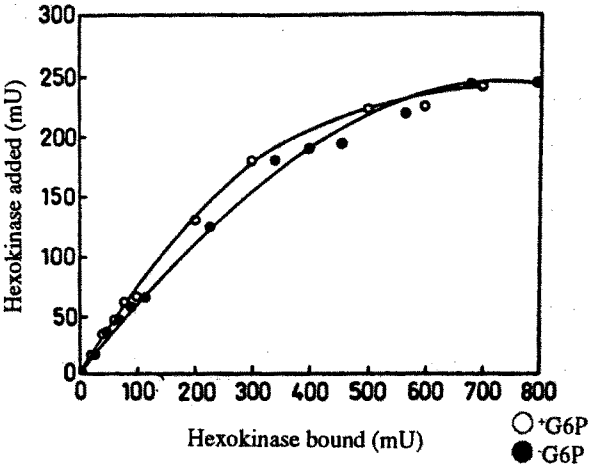

Figure 6. Titration of HK-depleted mitochondria with rat brain hexokinase. Indicated amounts of rat brain hexokinase (mU) were added to HK-depleted rat brain mitochondria (0.26 mg protein) in the SHT medium containing 2 mM $MgCl_2$. After incubation for 15 min at 28°C and centrifugation for 10 min at 40000 × g, the remaining activity in the supernatant fluid and bound activity in pellet were determined, in the presence, (○), and in the absence of G6P (●). G6P in enzyme solution was utilized by the addition of NADP+ and G6PD.

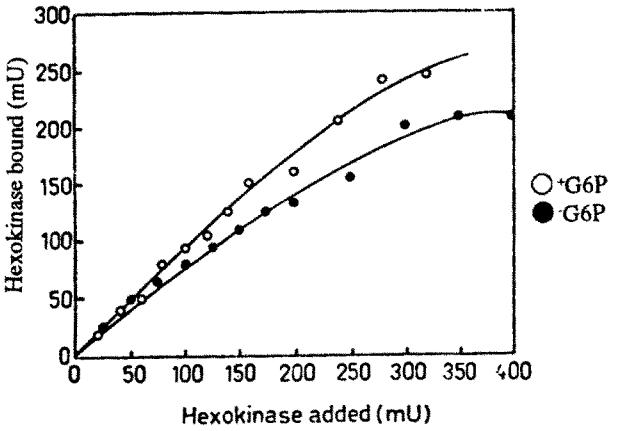

Figure 7. Titration of Sepharose-lipid particles with rat brain hexokinase. Indicated amounts of rat brain hexokinase (mU) in SHT medium were added to 50 µl of Sepharose-lipid suspension which had been equilibrated in SHT medium. After incubation for 10 min at room temperature and centrifugation for 10 min at 13500 rpm, activity remaining in supernatant and bound activity were determined, in the presence (○) and absence (●) of G6P.

carried out at 4°C. Also indicated are the results obtained using mitochondria (with bound HK) immobilized on Fractosil-1000, a porous form of silica. This experiment was carried out by first washing the two columns with SHT medium corresponding to 50 × column volume and demonstrating retention of mitochondria by assaying for marker enzymes (please see the Materials and Methods section). As indicated, both columns could be used in a continuous form.

Discussion

In addition to electrostatic interactions, hydrophobic interactions play an important role in the process of binding HK to the surface of the outer mitochondrial membrane. Based on the original suggestion made by Rose and Warms [28], G6P may shift the enzyme conformation to weaken the hydrophobic interactions with the membrane thereby causing its release.

It has been demonstrated that only types I and II hexokinases which contain the hydrophobic N-terminal tail can bind to mitochondria [10,25]. However, in the absence of porin, the presence of this segment is not sufficient for binding [33]. Recently, De Pinto *et al.* [6] suggested that insertion of this portion of enzyme molecule into the hydrophobic core of the membrane may place a positively charged Lysine-15 residue in a desirable position for electrostatic interaction with the N,N-dicyclohexyl carbodiimide (DCCD)-reactive, Glu-72 residue of porin. As indicated in our present report, the chymotrypsin-cleaved enzyme which lacks the hydrophobic N-terminal segment is not adsorbed on Sepharose-lipid matrix. On the other hand, G6P enhanced adsorption of HK to matrix. In other words, G6P strengthens the hydrophobic interactions on the artificial matrix rather than weakening them. It is, therefore, suggested that the change in the orientation of the hydrophobic tail brought about by G6P is the real cause of alteration in the mode of association of the enzyme to both adsorbents. These points are schematically presented in Figure 11. In preparation of this schematic drawing, the information provided in a similar presentation [36] was used.

Mg^{++} and spermine antagonize the solubilizing effect of G6P on the mitochondria-bound enzyme. According to Kurokawa *et al.* [15], these reagents shift the orientation of the enzyme to the original state preceding G6P effect. Similarly, during the adsorption of enzyme on Sepharose-

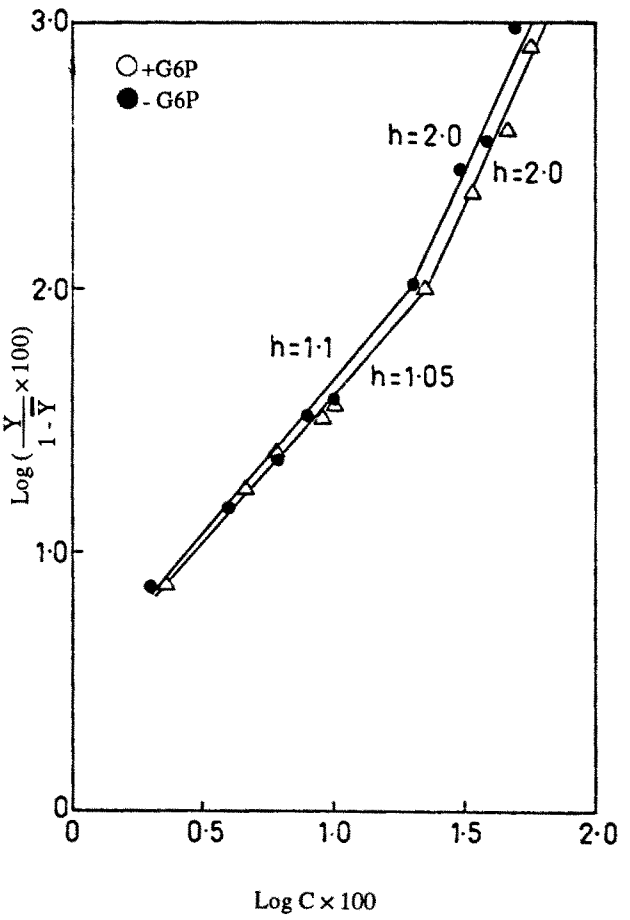

Figure 8. Hill plots of the binding of hexokinase to rat brain mitochondria in the presence and absence of G6P. C is the amount of added hexokinase (units) and Y represents fractional saturation. The values for saturation of mitochondria with the enzyme were extrapolated from the binding plots (see Fig. 6) to be (0.97 U/mg mitochondrial protein) and (0.98 U/mg mitochondrial protein).

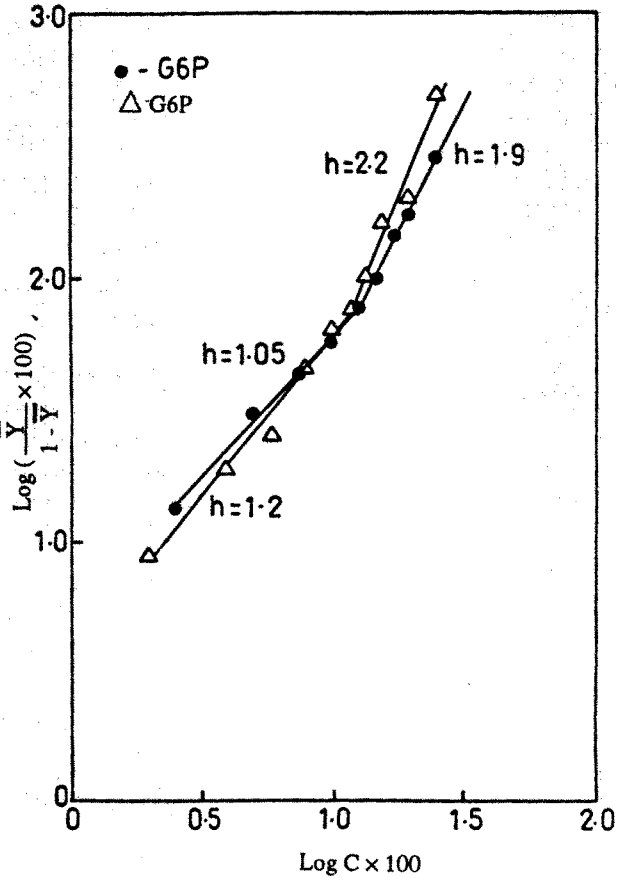

Figure 9. Hill plots of the adsorption of hexokinase on Sepharose-lipid suspension in the presence and absence of G6P. C and Y represent the amounts of added hexokinase and fractional saturation, respectively as noted in Figure 8. The values for saturation of the gel with the enzyme were extrapolated from the binding plots (see Fig. 7) and were found to be 4.9 and 4.1 unit/1ml packed Sepharose-lipid, respectively.

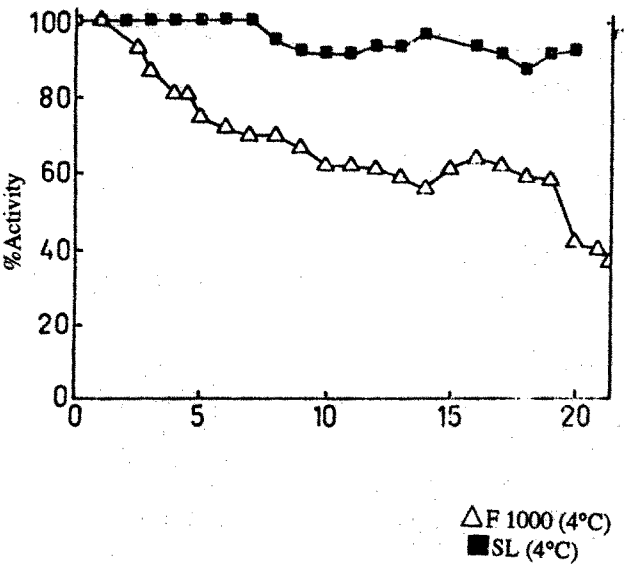

Figure 10. Continuous catalytic operation of mitochondrially-bound and Sepharose-lipid-immobilized hexokinase. Suspension of rat brain mitochondria (2.5 mg as protein) with the bound HK activity of 1 unit/ml, in SHT medium, was loaded on the column containing 1ml of packed Fractosil-1000. In a similar set of conditions, 1.0 ml of solubilized HK in SHT medium (0.75 units) was loaded on another column that contained 1.0 ml of packed Sepharose-lipid matrix. Both columns were equilibrated with appropriate amounts of SHT medium. Continuous catalytic activity of HK was then determined in each column for at least 20 hours as described in the Materials and Methods section. In both cases, enzyme assay mixture was passed through the columns at a constant flow rate of 0.4 ml/min.

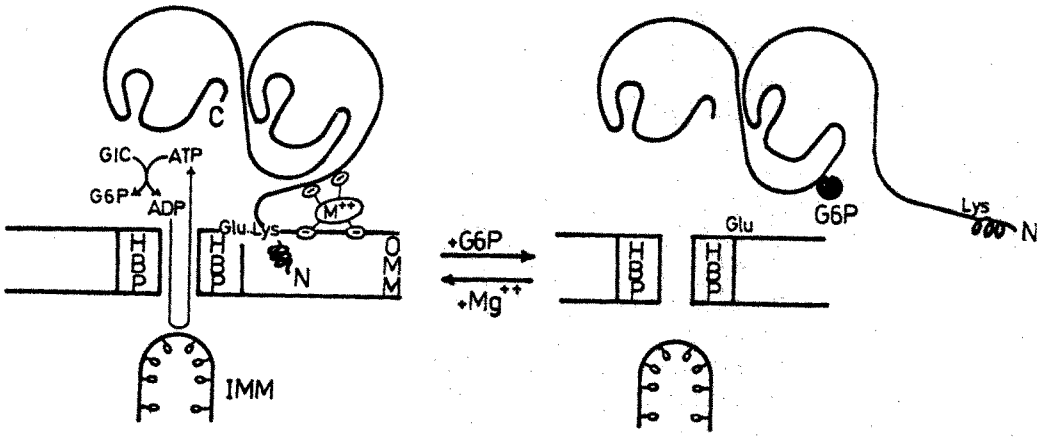

Figure 11. Schematic representation of the interaction of hexokinase with the outer mitochondrial membrane (OMM) and the role of G6P. For further details please see the text. IMM = Inner mitochondrial membrane; N = N-terminal; C = C-terminal; HBP = Hexokinase binding protein.

lipid, Mg^{++} and spermine can alter the orientation of the hydrophobic tail to reverse the G6P-induced enhancement. Thus, we conclude that Mg^{++} facilitates the ionic interactions that promote the N-terminal segment to achieve a position whereby a specific residue (probably Lys-15), can interact with Glu-72 residue of porin and, by maintaining this position, the segment can be inserted into the hydrophobic core of the membrane.

As discussed previously [21,22], immobilization of enzymes by adsorption to artificial matrices may provide useful models for *in vivo* processes. Accordingly, in the present study, it is concluded that release and/or binding of HK to the outer mitochondrial membrane and Sepharose-lipid matrix are the results of the same phenomenon.

Xie and Wilson [40] first reported that at least a portion of mitochondria-bound HK in rat brain is tetrameric and some of the porin molecules are surrounded by four molecules of HK. Later Wicker *et al.* (1993) estimated four HK molecules per porin in agreement with the results of Xie and Wilson. Titration curves and related Hill-plots in this paper for the binding of enzyme to mitochondria and Sepharose-lipid indicate positive cooperativity at high enzyme concentration which might point to the importance of protein-protein interactions for binding.

References

1. Adams, V., Bosch, W., Schlegel, J., Wallimannt, T. and Brdiczka, D. Further characterization of contact sites from mitochondria of different tissues: Topology of peripheral kinases. *Biochimica et Biophysica Acta*, **981**, 213-225, (1989).
2. Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K. and McCabe, E.R.B. Porin interaction with hexokinase and glycerolkinase: Metabolic microcompartmentation at the outer mitochondrial membrane. *Biochemical Medicine and Metabolic Biology*, **45**, (3), 271-291, (1991).
3. Brdiczka, D. Contact sites between mitochondrial envelope membranes: structure and function in energy and protein transfer. *Biochim. Biophys. Acta*, **1071**, 291-312, (1991).
4. Cesar, M.C. and Wilson, J.E. Application of double isotopic labelling method to a study of the interaction of mitochondria bound rat brain hexokinase with intra mitochondrial compartments of ATP generated by oxidative phosphorylation. *Arch. Biochem. Biophys*, **324**, (1), 9-14, (1995).
5. Chou, A.C. and Wilson, J.E. Hexokinase of rat brain. *Methods in Enzymology*, **XLII**, 20-25, (1975).
6. De Pinto, V., Al Jamal, J.A. and Palmieri, F. Location of the DCCD-reactive Glu residue in the bovine heart mitochondrial porin. *J. Biol. Chem.*, **268**, 12977-12982, (1993).
7. Faulkner, A. and Jones, C.T. Hexokinase isoenzymes in tissues of the adult and developing guinea-pig. *Arch. Biochem. Biophys.*, **175**, 477-486, (1976).
8. Felgner, P.L., Messer, J.L. and Wilson, J.E. Purification of a Hexokinase-binding protein from the outer mitochondrial membrane. *J. Biol. Chem.*, **254**, 4946-4949, (1979).
9. Fiek, C., Benz, R., Roos, N. and Brdiczka, D. Evidence for identity between the hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria. *Biochimica et Biophysica Acta*, **688**, 429-440, (1982).
10. Gelb, B.D., Adams, V., Jones, S.N., Griffin, L.D., MacGregor, G.R. and McCabe, E.R.B. Targeting of hexokinase-I to liver and hepatoma mitochondria. *Proc. Natl. Acad. Sci., USA*, **89**, 202-206, (1992).
11. Grossbard, L. and Schimke, R.T. Multiple hexokinases of rat tissues: Purification and comparison of soluble forms. *J. Biol. Chem.*, **241**, (15), 3546-3560, (1966).
12. Kabir, F. and Wilson, J.E. Mitochondrial hexokinases in brains of various species: Differences in sensitivity to solubilization by glucose-6-phosphate. *Arch. Biochem. Biophys.*, **300**, (2), 641-650, (1993).
13. Katzen, H.M., Soderman, D.D. and Wiley, C.E. Multiple forms of hexokinase: Activities associated with subcellular particulate and soluble fractions of normal and streptozotocin diabetic rat tissues. *J. Biol. Chem.*, **245**, (16), 4081-4096, (1970).
14. Kosow, D.P. and Rose, I.A. Ascites tumor mitochondrial hexokinase II: Effect of binding on kinetic properties. *Ibid.*, **243**, (13), 3623-3630, (1968).
15. Kurokawa, M., Yokoyama, K. and Ishibashi, S. Polyamines stimulate the binding of hexokinase type II to mitochondria. *Biochimica et Biophysica Acta*, **759**, 92-98, (1983a).
16. Kurokawa, M., Yokoyama, K., Kaneko, M. and Ishibashi, S. Difference in hydrophobicity between mitochondria-bindable and non-bindable forms of hexokinase purified from rat brain. *Biochem Biophys. Res. Commun.*, **115**, 1101-1107, (1983b).
17. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680-686, (1970).
18. Linden, M., Gellerfors, P. and Nelson, B.D. Pore protein and the hexokinase-binding protein from the outer membrane of rat liver mitochondria are identical. *FEBS Letters*, **141**, (2), 189-192, (1982).
19. McCabe, E.R.B. Microcompartmentation of energy metabolism at the outer mitochondrial membrane: Role in diabetes mellitus and other diseases. *J. Bioenerg. Biomembr.*, **26**, (3), 317-325, (1994).
20. Nemat-Gorgani, M. and Karimian, K. Non-ionic adsorptive immobilization of proteins to palmityl-substituted Sepharose-4B. *Eur. J. Biochem.*, **123**, 601-610, (1982).
21. Nemat-Gorgani, M. and Karimian, K. Enzyme immobilization on palmityl-Sepharose. *Biotechnology and Bioengineering*, **XXV**, 2617-2629, (1983).
22. Nemat-Gorgani, M., Karimian, K. and Massih, A.R. Effect of salt concentration on binding of proteins to a non-ionic adsorbent. *Experimentia*, **40**, 81-83, (1984).
23. Nemat-Gorgani, M. and Wilson, J.E. Acidic phospholipids may inhibit rat brain hexokinase by interaction at the nucleotide binding site. *Arch. Biochem. Biophys.*, **236**, (1),

220-227, (1985).

24. Nemat-Gorgani, M. and Wilson, J.E. Rat brain hexokinase: Location of the substrate nucleotide binding site in a structural domain at the C-terminus of the enzyme. *Ibid.*, **251**, (1), 97-103, (1986).
25. Polakis, P.G. and Wilson, J.E. An intact hydrophobic N-terminal sequence is critical for binding rat brain hexokinase to mitochondria. *Ibid.*, **236**, (1), 328-337, (1985).
26. Polakis, P.G. and Wilson, J.E. Proteolytic dissection of rat brain hexokinase: Determination of the cleavage pattern during limited digestion with trypsin. *Ibid.*, **234**, (2), 341-352, (1984).
27. Rickwood, D., Wilson, M.T. and Darley-Usmar, V.M. Isolation and characteristics of intact mitochondria. In *Mitochondria, a practical approach*, pp. 1-16. IRL press, (1987).
28. Rose, I.A. and Warms, J.V.B. Mitochondrial hexokinase: Release, rebinding and location. *J. Biol. Chem.*, **242**, (7), 1635-1645, (1967).
29. Schirch, D.M. and Wilson, J.E. Rat brain hexokinase: Amino acid sequence at the substrate hexose binding site is homologous to that of yeast hexokinase. *Arch. Biochem. Biophys.*, **257**, (1), 1-12, (1987).
30. Schnaitman, C., Erwin, V.G. and Greenawalt, W. The submitochondrial localization of monoamine oxidase. *J. Cell. Biol.*, **32**, 719-735, (1967).
31. Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. An electron-transport system associated with the outer membrane of liver mitochondria. *Ibid.*, **32**, 415-438, (1967).
32. White, T.K. and Wilson, J.E. Isolation and characterization of the discrete N- and C-terminal halves of rat brain hexokinase: Retention of full catalytic activity in the isolated C-terminal half. *Arch. Biochem. Biophys.*, **274**, (2), 375-393, (1989).
33. Wilson, J.E., Messer, J.L. and Felgner, P.L. Isolation of a hexokinase binding protein from the outer mitochondrial membrane. *Methods in Enzymology*, **97**, 469-475, (1983).
34. Wilson, J.E. Ambiquitous enzymes: Variation in intracellular distribution as a regulatory mechanism. *Trends Biochem. Sci.*, **3**, 124-125, (1978).
35. Wilson, J.E. Rapid purification of mitochondrial hexokinase from rat brain by a single affinity chromatography step on Affi-Gel Blue. *Preparative Biochemistry*, **19**, (1), 13-21, (1989).
36. Wilson, J.E. Brain hexokinases. In *Reviews of physiology, biochemistry and pharmacology*, Vol. 126, pp. 65-197. Springer-Verlag, (1995).
37. Wilson, J.E. Regulation of mammalian hexokinase activity. In *Regulation of carbohydrate metabolism*, Vol. 1, (ed. R. Beitner), pp. 45-85. CRC, Boca Raton, (1985).
38. Wilson, J.E. Brain hexokinase. *J. Biol. Chem.*, **243**, (13), 3640-3647, (1968).
39. Xie, G. and Wilson, J.E. Rat brain hexokinase: The hydrophobic N-terminus of the mitochondria bound enzyme is inserted in the lipid bilayer. *Arch. Biochem. Biophys.*, **267**, (2), 803-810, (1988).
40. Xie, G. and Wilson, J.E. Tetrameric structure of mitochondria bound rat brain hexokinase: A cross-linking study. *Ibid.*, **276**, (1), 285-293, (1990).