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PROPERTIES OF THE GLUCOSE 6-PHOSPHATE-SOLUBILIZED BRAIN HEXOKINASE

EVIDENCE FOR A LIPOPROTEIN COMPLEX

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SUMMARY

1. Glucose 6-phosphate (Glc-6-*P*)-solubilized brain hexokinase is part of a large aggregate as shown by its appearance in the void volume upon Sephadex G-200 and Sepharose 4B column chromatography.

2. Thin-layer chromatography of chloroform–methanol (2:1, v/v) extracts indicate the presence of cholesterol, phosphatidylethanolamine and, tentatively, one or more neutral lipid components in the Glc-6-*P* supernatant but not in a control supernatant prepared by incubation in the absence of Glc-6-*P* and lacking significant hexokinase activity.

3. The Glc-6-*P* supernatant has a phosphorus to protein ratio of 9 ± 2 μg P/mg protein which is in rough agreement with the values reported, in the literature for brain mitochondrial membranes.

4. Mitochondria, rendered lipid deficient by treatment with cold 10 % aqueous acetone, lost 80–95 % of their total hexokinase activity and this activity could be partially restored by incubation with phosphatidylethanolamine but not with phosphatidylcholine nor phosphatidylserine. Complete recovery of activity was obtained in preparations which had lost no more than 60 % of their activity.

5. Digitonin prevents the $MgCl_2$-mediated rebinding of hexokinase to the mitochondrial membrane at concentrations which do not solubilize hexokinase from intact mitochondria.

INTRODUCTION

The association of 75–90 % of the total hexokinase activity found in brain with the mitochondrial fraction obtained from cell free extracts is well documented[1–3]. Furthermore, its presence on the outer mitochondrial membrane has been indicated by three different methods. These are the ease with which it can be solubilized and rebound by a variety of reagents including one of its products, glucose 6-phosphate (Glc-6-*P*) (refs. 4 and 5), direct assay of the inner and outer membranes of brain mitochondria[6] and the demonstration of binding sites on the outer but not the inner membrane of rat brain mitochondria[7]. Tentative evidence is available to indicate that

hexokinase is also associated with mitochondria in the intact cell[8], however, this point still remains to be confirmed in the electron microscope.

The physiological significance of the unique location of hexokinase on the mitochondrial membrane as compared with all the other glycolytic enzymes which are found in the soluble fraction of brain homogenates has been the subject of much recent work. CHANCE AND HESS[9] first suggested that there is a direct shuttle of ADP from the hexokinase system to the respiratory chain of mitochondria. Evidence obtained by GUMAA AND MCLEAN[10] indicates that this is true in ascites tumor cells. Upon the addition of glucose to Ehrlich ascites tumor cells, there is a rapid and linear rate of ATP utilization which largely parallels the rate of ATP regeneration. After 20 sec there is a sharp decline which could possibly be correlated with the solubilization of hexokinase by accumulated Glc-6-*P* and its subsequent inhibition by Glc-6-*P*. This is in agreement with the evidence that soluble ascites tumor cell hexokinase is more susceptible to Glc-6-*P* inhibition than membrane bound hexokinase[11]. MOORE AND JÖBSIS[12] have recently reported a similar Glc-6-*P* inhibited stimulation of mitochondrial respiration by glucose in rat brain. Thus the unique association of hexokinase with brain and ascites tumor cell mitochondria may provide an important means of regulating respiration in these tissues by providing a readily available source of ADP. In addition, the rapid and specific solubilization of mitochondrial hexokinase by Glc-6-*P* may provide a secondary mechanism for controlling respiration in brain mitochondria. The present study of some properties of the Glc-6-*P*-solubilized brain hexokinase should provide further insight into this secondary mode of control.

MATERIALS

The following materials were obtained commercially: Glc-6-*P*, Glc-6-*P* dehydrogenase (Type 5), ATP, $NADP^+$, cytochrome *c* (Type 3) and peroxidase (Type 2) from Sigma Chemical Co., St. Louis, Mo.; Sephadex gels and columns from Pharmacia, Uppsala, Sweden; bovine plasma albumin from Armour Pharmaceutical Co., Kankakee, Ill.; digitonin A grade from Calbiochem, Los Angeles, Calif.; Chrom AR 500 from Mallinckrodt Chemical Works, St. Louis, Mo.; ninhydrin aerosol spray reagent from Nutritional Biochemicals Corp., Cleveland, Ohio; chromatographically pure bovine lecithin, phosphatidylethanolamine, phosphatidylserine and cardiolipin, from Supelco, Bellefonte, Pa.; chloroform and methanol, spectranalyzed from Fisher Scientific Co., Pittsburgh, Pa. All other reagents of analytical grade were obtained from Fisher Scientific Co., Pittsburgh, Pa.

METHODS

Preparation of the Glc-6-P supernatant

Rat brain mitochondria, prepared by the method of OZAWA *et al.*[13] were incubated, with gentle agitation, at 30°, in 0.3 M mannitol–0.1 mM EDTA–0.8 mM Glc-6-*P* (pH 7.4). Each incubation mixture contained 3 mg of mitochondrial protein and 1 unit of hexokinase activity in a total volume of 2 ml. A more concentrated hexokinase preparation was sometimes obtained by incubating 18 mg of mitochondrial protein and 6 units of hexokinase activity in a total of 2 ml of 4.8 mM Glc-6-*P*. However, this resulted in the solubilization of only 60–70 % of the hexokinase activity.

It also lowered the specific activity of solubilized hexokinase from 6 to 3 units/mg protein. After 10 min the mitochondria were sedimented at 105000 × *g* for 1 h in the Spinco Model L centrifuge (rotor No. 40) and both the sediment and the Glc-6-*P* supernatant saved for assay. Control supernatants were prepared in the same way except that Glc-6-*P* was omitted from the incubation mixture.

Measurement of hexokinase activity

Hexokinase activity was measured in a system in which Glc-6-*P* production was coupled to NADPH formation in the presence of Glc-6-*P* dehydrogenase. The procedure used is described by SCHWARTZ AND BASFORD[14]. One unit of enzyme activity is defined as the formation of 1 μmole of Glc-6-*P* per min at 25°. Dilutions for assay were made with 0.3 M mannitol–0.1 mM EDTA. There was a short burst of NADPH formation when the assay was performed in the presence of Glc-6-*P*, however, after 30 sec the assay proceeded normally. In determining the K_m of hexokinase for ATP, Glc-6-*P* was always removed first by passage through a short column of Sephadex G-25 in a procedure devised for desalting small volumes[14].

Determination of protein

Protein was determined by the method of LOWRY *et al.*[15] as described by SCHWARTZ AND BASFORD[14]. The buffer composition of the standard bovine plasma albumin solution was always adjusted to the same composition as the sample since mannitol has a significant effect on the standard curve. Alternatively protein was estimated by its absorbance at 280 nm.

Determination of phosphorus

Phosphorus was determined by the ultra-micro heating method of BARTLETT[16] after dialysis overnight against deionized water to remove buffer salts.

Determination of molecular weights

Molecular weights were determined, using Sephadex G-200, from the straight line obtained by plotting the ratio of the elution volume to the void volume *versus* the log of the molecular weight. The following proteins were used as standards: cytochrome *c*, 13000 (ref. 17); peroxidase, 40200 (ref. 18); bovine serum albumin, 70000 (ref. 19). The void volume was determined experimentally with blue dextran. The dimensions of the column used were 43 cm × 2.5 cm and the flow rate was usually 10 ml/h.

Digitonin was prepared according to the method described by SCHNAITMAN *et al.*[20].

Preparation of lipid extracts

Lipid extracts were prepared by shaking the Glc-6-*P* or control supernatant with chloroform–methanol (2:1, v/v) as described by RECKNAGEL AND GHOSHAL[21]. The final total lipid extract in chloroform was concentrated under a stream of N_2 and used for thin-layer chromatography.

Thin-layer chromatography

The chromatography was done using commercially available Chrom AR 500, silicic acid-impregnated glass fibre. The solvent system used was chloroform–methanol

(85:15, v/v). Spots were routinely observed by illumination with short-wave ultraviolet light, and exposure of the dried chromatograms to I_2 vapor. Cholesterol was identified by the Lieberman–Burchard reaction[22] and by comparison with a cholesterol standard. Phosphatidylethanolamine was identified by reaction with ninhydrin and the molybdenum blue spray reagent[23]. Commercially available phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and cardiolipin, all of very high purity were used as chromatographic standards. The solvent system used resulted in clear resolution of these standards.

Preparation of phospholipid dispersions

After evaporation to dryness under a stream of N_2, the sample was resuspended in 0.02 M Tris·acetate–1 mM EDTA (pH 8) at a concentration of 12.5 mg lipid per ml and sonicated in a Branson probe sonifier with a micro tip. For reconstitution experiments the samples were incubated with cold aqueous acetone-extracted mitochondria for at least 15–20 min in the cuvette before the addition of the hexokinase assay medium.

Cold acetone extraction

Extraction for 8 min at a final concentration of 10 % water in acetone was done according to the method described by FLEISCHER AND FLEISCHER[24]. The mitochondria contained 10 mg of protein and 3 units of hexokinase activity in 1 ml.

RESULTS

Total phosphorus content and specific activity of hexokinase in the Glc-6-P supernatant

Table I summarizes some of the properties of Glc-6-*P*-solubilized hexokinase. The specificity of action of the Glc-6-*P* can be realized by the following comparison. The specific activity of hexokinase in mitochondria as prepared by the method of OZAWA *et al.*[13] is usually 0.2–0.3 unit/mg of mitochondrial protein. After mild incubation for 10 min at 30° with 0.8 mM Glc-6-*P* in isotonic medium (0.3 M mannitol–0.1 mM EDTA) 90–100 % of the hexokinase activity but only 5 % of the total mitochondrial protein is released into the high-speed supernatant fraction (105000 × *g* for 1 h, Spinco rotor No. 40). Continuing the centrifugation for 24 h did not change this result. The specific activity of hexokinase in the Glc-6-*P* supernatant is usually

TABLE I

PROPERTIES OF MITOCHONDRIAL HEXOKINASE ELUTED WITH 0.8 mM Glc-6-*P*

Centrifugation for 24 h at 105000 × *g* in isotonic medium	Non-sedimentable
Specific activity	5–6 units/mg protein
Phosphate/protein ratio	9 μg P_i/mg protein
Protein concentration	70 μg/ml
K_m for ATP	0.93 mM (50 μM glucose)
	1.8 mM (12 mM glucose)
Sephadex G-200 chromatography (0.3 M mannitol–0.1 mM EDTA)	Void volume

5–6 units/mg protein. This must be compared with the purest preparation of soluble brain hexokinase yet reported which has a specific activity of 80 units/mg protein after extensive purification by detergent treatment and DEAE-cellulose chromatography[14]. Solubilization by Glc-6-*P* is not, however, suitable for the preparation of large quantities of hexokinase as is the procedure of SCHWARTZ AND BASFORD[14] because of the very low starting protein concentrations (1.5–2 mg/ml) needed to attain 90 % solubilization. Increasing the starting protein concentration results in a decrease in the percent solubilized as well as the specific activity of hexokinase (see METHODS). Therefore, although the Glc-6-*P* supernatant fraction contains 90–100 % of the total hexokinase activity in the isolated mitochondria, the protein concentration is only 70 μg/ml. This represents 140 μg/3 mg of mitochondrial protein in each incubation. Of this, only 4 % can be assigned to hexokinase, assuming a maximal specific activity of the pure enzyme of 80 units/mg. As a result, attempts to visualize the hexokinase particle with the electron microscope and attempts to obtain a purer hexokinase preparation have failed.

The phosphorus content of the Glc-6-*P* supernatant was determined after dialysis overnight against deionized water to remove buffer salts. The removal of Glc-6-*P* was confirmed spectrophotometrically by the addition of 0.1 ml of the dialyzed sample to the hexokinase assay system in the absence of glucose. There was no change in the absorbance at 340 nm indicating that $NADP^+$ was not being reduced. The average value obtained (4 determinations) was 9 ± 2 μg/mg protein. The phosphorus to protein ratio in a control supernatant obtained by incubating mitochondria at 30° for 15 min in the absence of Glc-6-*P* was 5 ± 1.5 μg/mg protein. In one experiment, the phosphorus determination was performed on lipid extracts of the control and Glc-6-*P* supernatants. The same results were obtained, indicating that the values obtained reflect the content of lipid phosphorus. There was no significant difference between the protein content of the control and Glc-6-*P* supernatant. These ratios should be compared with the phosphorus to protein ratio reported for beef brain mitochondrial membranes, 13 μg P/mg protein[25] and that for rat brain mitochondrial membranes, 17 μg/mg dry weight of tissue. The dry weight of tissue is determined as acid insoluble dry matter[26].

Determination of the K_m for ATP of the Glc-6-P supernatant hexokinase

Since it has been shown that pure, detergent solubilized brain mitochondrial hexokinase, as prepared by the method of SCHWARTZ AND BASFORD[14] and brain mitochondrial hexokinase which is still in association with the mitochondrial membrane differ in their K_m values for ATP (refs. 14, 27 and 28), it was of interest to determine the K_m for ATP of the hexokinase present in the Glc-6-*P* supernatant. The K_m for ATP was found to be the same as that for the detergent solubilized form, *i.e.* 0.93 and 1.8 mM at glucose concentrations of 0.05 and 12 mM, respectively.

Sephadex G-200 chromatography of the Glc-6-P supernatant

Fig. 1 shows the results obtained when 4 ml of the Glc-6-*P* supernatant containing 2 units of hexokinase activity and 0.3 mg of protein is chromatographed on Sephadex G-200. The eluting buffer was 0.3 M mannitol–0.1 mM EDTA. All of the hexokinase activity, all of the 280-nm absorbing material, and all of the phosphorus (not shown) was recovered in the void volume of the column. The phosphorus to

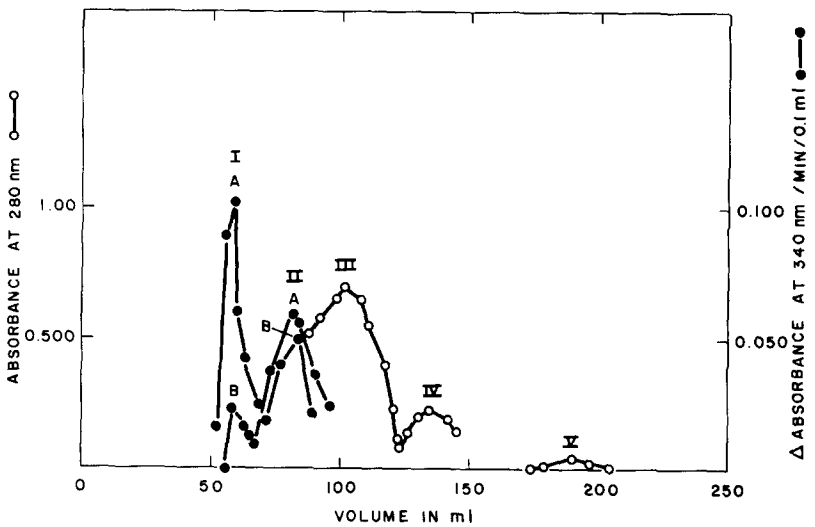

Fig. 1. Sephadex G-200 column chromatography. The elution pattern shown is a composite of several different experiments using the same column of dimensions 2.5 cm × 43 cm and having a void volume of 58 ml. The eluting buffer was 0.3 M mannitol–0.1 mM EDTA (pH 7.5). Peaks I and II were monitored by hexokinase assay. Peak I had an elution volume of 58 ml, the same as the void volume. Peak IA is the Glc-6-*P*-solubilized hexokinase, and IB is the hexokinase present in the high-speed supernatant of brain homogenates. Peak II had an elution volume of 78 ml. Peak IIA is yeast hexokinase and Peak IIB is the hexokinase present in the high-speed supernatant of liver homogenates. Peaks III, IV and V represent the following standard proteins: III, bovine plasma albumin, elution volume 101.5 ml; IV, peroxidase, elution volume 136 ml; and V, cytochrome *c*, 190 ml. The bed volume was 209 ml.

protein ratio of the sample after Sephadex chromatography was essentially unchanged. It was found necessary to collect the hexokinase fractions in plastic tubes due to extensive activity losses upon adsorption to glass. When essentially complete recovery of hexokinase activity was obtained, the specific activity was increased from 5–6 units/mg in the applied sample to 12 units/mg in the peak tube. The molecular weights of yeast hexokinase and rat liver supernatant hexokinase were calculated as described under METHODS from the observed elution volumes of the standards shown. The value obtained, 100000, agrees with that already quoted in the literature[29,30]. Chromatography using Sepharose 4B, which has an exclusion limit of $20 \cdot 10^6$ for proteins again resulted in the elution of all the hexokinase activity found in the Glc-6-*P* supernatant in the void volume. Column chromatography is only used here as a rough indication of the size of solubilized hexokinase as molecular weight determination using Sephadex and Sepharose is only applicable to globular proteins. Since it has been shown that approx. 10–25 % of the brain hexokinase activity appears in the soluble fraction of brain homogenates, it was of interest to chromatograph a sample of brain supernatant hexokinase on Sephadex G-200. Previously the molecular weight of brain supernatant hexokinase, purified by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose and hydroxylapatite chromatography, was determined by sucrose gradient centrifugation to be 100000 (ref. 29). However, after precipitation of the high-speed supernatant, obtained from a rat brain homogenate, in 50 % $(NH_4)_2SO_4$ and chromatography on Sephadex G-200 using 0.3 M mannitol–0.1 mM EDTA as the eluting buffer, all the hexokinase

activity was again recovered in the void volume (Fig. 1). It is possible that the difference in purification procedures is responsible for the difference in molecular weights.

Thin-layer chromatography

Thin-layer chromatography of the chloroform–methanol (2:1, v/v) extract of either the Glc-6-*P* supernatant or the void volume obtained by chromatography of the Glc-6-*P* supernatant on Sephadex G-200, always gave just four spots, three of which were identified as lipids. The results are shown in Fig. 2. Spot 1, which traveled with the solvent front, and did not yield a yellow color upon exposure to I_2 vapor, was unidentified. Spot 2 gave a yellow color with I_2 but did not react with the molybdenum blue spray reagent. It had the same R_F as an oleic acid standard. It is tentatively classified as one or more neutral lipid components which could include mono- or diglycerides as well as fatty acid, however, the presence of glycolipid cannot as yet be ruled out. Spot 3 was identified as cholesterol by the Lieberman–Burchard reaction and by direct comparison with an authentic sample of cholesterol. Spot 4 was identified as phosphatidylethanolamine by reaction with ninhydrin, the molybdenum blue spray reagent and direct comparison with a phosphatidylethanolamine standard. Thin-layer chromatography of the chloroform–methanol (2:1, v/v) extract of a control supernatant, obtained by incubating mitochondria at 30° for 10 min in the absence of Glc-6-*P* as described under METHODS, did not reveal the presence of detectable amounts of lipid. Thin-layer chromatography of the lipid extract of the void volume of a Sephadex G-200 column obtained from the chromatography of the

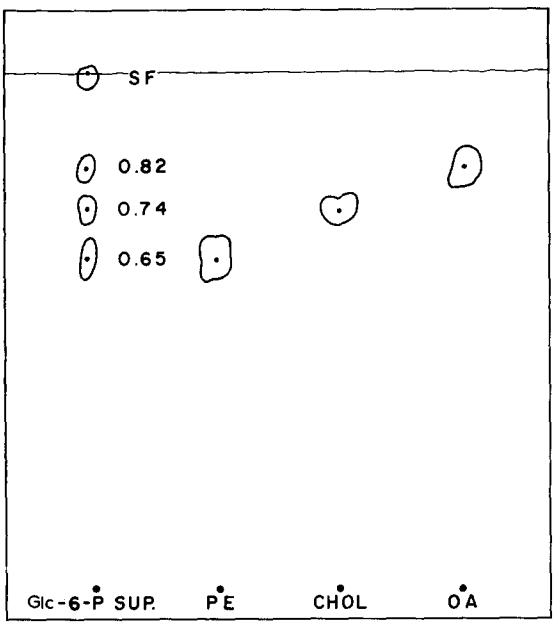

Fig. 2. Thin-layer chromatography of a chloroform–methanol (2:1, v/v) extract of the Glc-6-*P* supernatant as compared with standard phosphatidylethanolamine (PE), cholesterol (CHOL) and oleic acid (OA). The spot at the solvent front (SF) was not identified. The R_F values shown were calculated from the center of each spot, and the spots were detected as described in the text. The developing solvent was chloroform–methanol (85:15, v/v).

Glc-6-*P* supernatant resulted in the appearance of the same four spots. This fact and the finding of a phosphorus/protein ratio of 8 μg P_i/mg protein in the void volume suggests that the presence of a high-molecular-weight hexokinase is not simply the result of protein aggregation.

Cold 10 % aqueous acetone extraction of mitochondria

Freshly prepared rat brain mitochondria lost 80–95 % of their hexokinase activity when extracted with cold aqueous acetone. Fig. 3 demonstrates the ability of phosphatidylethanolamine, but not phosphatidylserine nor phosphatidylcholine, to partially restore hexokinase activity to lipid deficient mitochondria. In preparations where not more than 60 % of the hexokinase activity had been lost, phosphatidylethanolamine completely restored hexokinase activity. The effect of cardiolipin on the restoration of activity to lipid deficient mitochondria could not be tested due to flocculation in the cuvette on the addition of cardiolipin. It would, however, not be expected to have a significant effect as the lipid-deficient mitochondria used in these experiments retain most of their original cardiolipin[24].

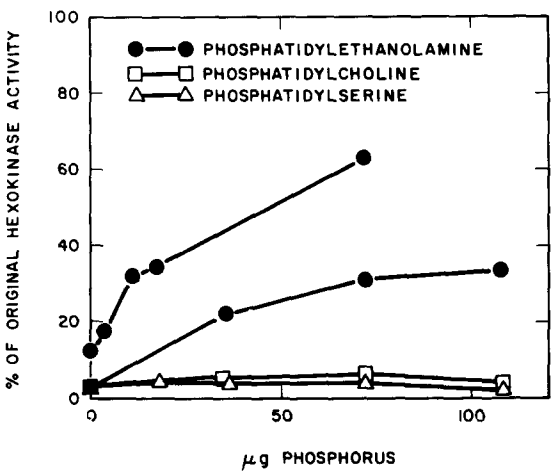

Fig. 3. The restoration of hexokinase activity to lipid-deficient mitochondria. Lipid-deficient mitochondria which had lost 80–95 % of their hexokinase activity by extraction in cold aqueous acetone were diluted for assay with 0.3 M mannitol–0.1 mM EDTA (pH 7.4). Incubations were carried out for 15–20 min at room temperature with the indicated amounts of phospholipid in the reaction cuvette before the addition of the hexokinase assay medium. The hexokinase activity of the mitochondria before treatment with acetone (9.3 nmoles/min per assay) is taken to be 100 %.

Effect of digitonin on the $MgCl_2$-mediated rebinding of the Glc-6-P-solubilized hexokinase to the mitochondrial membrane

As can be seen in Fig. 4, 6 mM $MgCl_2$ is sufficient to achieve maximal rebinding of hexokinase to the mitochondrial membrane. This was first shown by ROSE AND WARMS[4] for ascites tumor cell mitochondria. However, as shown in Fig. 4, the addition of small amounts of digitonin, 0.11 mg digitonin per mg mitochondrial protein, prior to the addition of 8 mM $MgCl_2$, completely prevents the rebinding of hexokinase to the membrane. As is also shown, the addition of digitonin to mitochondria that have not been treated with Glc-6-*P* does not result in the release of any significant amounts

of hexokinase even at concentrations as high as 0.78 mg digitonin per mg mitochondrial protein. It is interesting to speculate that this result might indicate a role for cholesterol in the binding of hexokinase to the mitochondrial membrane. It was previously reported[4] that the addition of chymotrypsin to an incubation medium containing solubilized hexokinase and ascites tumor particles, prevented the $MgCl_2$-induced rebinding of hexokinase to the mitochondrial membrane. However, chymotrypsin was found to have no effect on the rebinding of brain hexokinase.

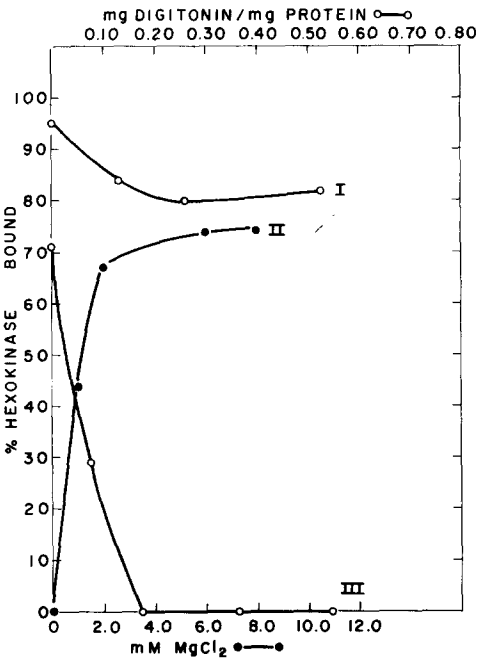

Fig. 4. Effect of digitonin on the rebinding of Glc-6-*P*-solubilized hexokinase to the mitochondrial membrane. Each incubation contains approx. 1 mg of mitochondrial protein. Curve I shows that the incubation of intact brain mitochondria with digitonin for 10 min at 30° at the indicated digitonin concentrations does not solubilize hexokinase to any appreciable extent. Curve II shows the rebinding effect of $MgCl_2$ 5 min after its addition to a suspension of mitochondria in 0.3 M mannitol–0.1 mM EDTA which has been incubated for 10 min with 0.8 mM Glc-6-*P* to solubilize hexokinase. Curve III shows the effect of digitonin on the $MgCl_2$-induced rebinding of Glc-6-*P*-solubilized hexokinase to the mitochondrial membrane. After incubation with 0.8 mM Glc-6-*P* for 5 min, digitonin was added in the indicated amounts and the incubation continued for 5 min. Then 8 mM $MgCl_2$ was added to each beaker and the incubation continued for 5 min more. The supernatants obtained by sedimenting the incubation mixtures at 105 000 × *g* for 1 h were assayed for hexokinase activity and the percent of hexokinase bound to the mitochondria was determined by the difference.

DISCUSSION

The results of others[31, 32, 29] stress the importance of recognizing the varied distribution of hexokinase isozymes found in all rat tissues studied and not referring to the hexokinase types simply as "brain hexokinase" or "liver hexokinase". However, it should be pointed out that most of these very interesting studies have been done

solely on the hexokinase activity found in the high-speed supernatant of tissue homogenates and, as yet, have not been extended to include a correlation between the association of hexokinase with particulate matter in different tissues and the isozyme pattern in these tissues. One recent report, however, has identified as Isozymes I and II, a portion of the hexokinase activity bound to the mitochondria of rat epididymal adipose tissue[33]. A study of this sort is complicated by the fact that hexokinase is easily solubilized by a number of agents which could be found in tissue homogenates. For example, ROSE AND WARMS[4] have presented evidence which suggests that liver hexokinase may be associated with mitochondrial structure *in vivo* but become solubilized by lysosomal enzymes during homogenization. Similarly, the studies of ROSE AND WARMS[4] and WILSON[5] show that the presence of various metabolites could influence the proportion of soluble and particulate hexokinase in tissue homogenates.

Most of the available evidence, however, indicates that brain mitochondrial hexokinase, which accounts for 90 % of the total hexokinase activity of brain tissue, is identical to the enzyme found in the high-speed supernatant of brain homogenates which has been shown by KATZEN AND SCHIMKE[31] to be composed almost entirely of Isozyme I. First, solubilized brain mitochondrial hexokinase is immunologically identical with soluble brain hexokinase[8]. Second, the K_m for glucose of mitochondrial brain hexokinase[27], detergent-solubilized mitochondrial hexokinase[14,28] and soluble brain hexokinase[31] are all substantially the same. The K_m for glucose is one of the criteria used to differentiate between the four hexokinase isozymes.

If it can be assumed that the majority of brain hexokinase is Isozyme I, then it would be very interesting to compare the molecular weights and lipid compositions of Isozyme I isolated from various rat tissues as this might indicate a previous association of Isozyme I with particulate material regardless of the possibility of solubilization by Glc-6-*P* during homogenization. On the other hand, the lipoprotein nature of brain hexokinase might be a property unique to this tissue and unrelated to isozyme types or it could simply be a result of the nonspecific interaction of Glc-6-*P*-solubilized hexokinase with lipid components released during the incubation procedure.

The phospholipid requirement for activity following the aqueous acetone extraction of mitochondria is not incompatible with the existence of a solubilized form of the enzyme, free from attached phospholipid. This has already been shown to be true for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase[34]. As already discussed by GREEN *et al.*[34] and LENAZ[35], the repeating units in a membrane are molecularized as opposed to disordered and aggregated. Lipids are specifically bound at certain sites of the repeating units so as to prevent the polymerization and loss of activity of membrane enzymes. If the enzymes are water solubilized, they no longer need lipid to prevent aggregation.

Finally, the results presented in this paper indicate that the incubation of brain mitochondria with Glc-6-*P*, the end-product inhibitor of hexokinase, results in the release of a large aggregate containing fully active hexokinase as well as phosphatidylethanolamine, cholesterol and neutral lipid. Further study is needed to determine whether this aggregate retains the structural organization it possessed as part of the brain mitochondrial membrane.

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REFERENCES

1 R. K. CRANE AND A. SOLS, *J. Biol. Chem.*, 203 (1953) 273.
2 M. K. JOHNSON, *Biochem. J.*, 77 (1960) 610.
3 R. E. BASFORD, W. L. STAHL, D. S. BEATTIE, H. R. SLOAN, J. C. SMITH AND L. M. NAPOLITANO, in M. M. COHEN AND R. S. SNIDER, *Morphological and Biochemical Correlates of Neural Activity*, Harper and Row, New York, N.Y., 1964, p. 192.
4 I. A. ROSE AND J. V. B. WARMS, *J. Biol. Chem.*, 242 (1967) 1635.
5 J. E. WILSON, *J. Biol. Chem.*, 243 (1968) 3640.
6 P. A. CRAVEN, P. J. GOLDBLATT AND R. E. BASFORD, *Biochemistry*, 8 (1969) 3525.
7 E. S. KROPP AND J. E. WILSON, *Biochem. Biophys. Res. Commun.*, 38 (1970) 74.
8 P. A. CRAVEN AND R. E. BASFORD, *Biochemistry*, 8 (1969) 3520.
9 B. CHANCE AND B. HESS, *Ann. N.Y. Acad. Sci.*, 63 (1956) 1008.
10 K. A. GUMAA AND P. MCLEAN, *Biochem. Biophys. Res. Commun.*, 36 (1969) 771.
11 D. P. KOSOW AND I. A. ROSE, *J. Biol. Chem.*, 243 (1968) 3623.
12 C. L. MOORE AND F. F. JÖBSIS, *Arch. Biochem. Biophys.*, 138 (1970) 295.
13 K. OZAWA, K. SETA, H. TAKEDA, K. ANDO, H. HARIDA AND C. ARAKI, *J. Biochem. Tokyo*, 59 (1966) 501.
14 G. P. SCHWARTZ AND R. E. BASFORD, *Biochemistry*, 6 (1967) 1070.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
16 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
17 H. THEORELL AND Å. ÅKESSON, *J. Am. Chem. Soc.*, 63 (1941) 1804.
18 H. THEORELL AND A. EHRENBERG, quoted by A. C. MAEHLY, *Methods Enzymol.*, 2 (1955) 801.
19 P. JOHNSON, *Annu. Rep. Prog. Chem.*, 43 (1948) 53.
20 C. SCHNAITMAN, V. G. ERWIN AND J. W. GREENAWALT, *J. Cell Biol.*, 32 (1967) 719.
21 R. O. RECKNAGEL AND A. K. GHOSHAL, *Exp. Mol. Pathol.*, 5 (1966) 413.
22 J. G. KIRCHNER, *Techn. Org. Chem.*, 12 (1967) 165.
23 J. C. DITTMER AND R. S. LESTER, *J. Lipid Res.*, 5 (1964) 126.
24 S. FLEISCHER AND B. FLEISCHER, *Methods Enzymol.*, 10 (1967) 406.
25 P. PARSONS AND R. E. BASFORD, *J. Neurochem.*, 14 (1967) 823.
26 L. A. BIRAN AND W. BARTLEY, *Biochem. J.*, 79 (1961) 159.
27 H. J. FROMM AND V. ZEWE, *J. Biol. Chem.*, 237 (1962) 1661.
28 M. COPLEY AND H. J. FROMM, *Biochemistry*, 6 (1967) 3503.
29 L. GROSSBARD AND R. T. SCHIMKE, *J. Biol. Chem.*, 241 (1966) 3546.
30 Y. M. RUSTUM, E. J. MASSARO AND E. A. BARNARD, *Fed. Proc.*, 28 (1969) 468.
31 H. M. KATZEN AND R. T. SCHIMKE, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1218.
32 H. M. KATZEN, *Adv. Enzyme Regul.*, 5 (1967) 335.
33 B. BORREBAEK, *Biochem. Med.*, 3 (1970) 485.
34 D. E. GREEN, D. W. ALLMANN, E. BACHMANN, H. BAUM, K. KOPACZYK, E. F. KORMAN, S. LIPTON, D. H. MACLENNAN, D. G. MCCONNELL, J. F. PERDUE, J. S. RIESKE AND A. TZAGOLOFF, *Arch. Biochem. Biophys.*, 119 (1967) 312.
35 G. LENAZ, *Ital. J. Biochem.*, 17 (1968) 129.