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ASSOCIATION OF GLYCOLYTIC ENZYMES WITH PARTICULATE FRACTIONS FROM NERVE ENDINGS

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Summary

Several glycolytic enzymes were observed to have between 40—90% of their activities associated with the particulate fractions of lysed nerve endings. The enzymes showing high particulate activity in lysed nerve endings were hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.13), glucosephosphate isomerase (EC 5.3.1.9), phosphofructokinase (EC 2.7.1.11), glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.27). With the exception of phosphofructokinase, 80% or more of the particle associated activity of each enzyme was solubilized by salt treatment indicating the association with particles was ionic. Sub-fractionation of lysed nerve endings showed hexokinase and fumarase (EC 4.2.1.2) had the highest specific activity in the same fractions which is consistent with observations indicating that hexokinase is associated with mitochondria. The other glycolytic zymes having high particulate activity, aldolase, glucosephosphate isomerase, phosphofructokinase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase, showed enrichment in fractions containing synaptosomal membranes, i.e. the fractions having highest specific activity of acetylcholinesterase (EC 3.1.1.7) and (Na^+ + K^+)-ATPase (EC 3.6.1.3).

Introduction

Intracellular coordination of glycolysis rather than random collision of substrates with the appropriate active sites of enzymes has been implied from theoretical calculations [1], however, supportive experimental evidence has been lacking. Several studies have shown that certain glycolytic enzymes, hexokinase [2—6], phosphofructokinase [4,7], aldolase [8—10] and pyruvate kinase [11,12] are bound to particulate fractions in brain homogenates. Glyceraldehyde-phosphate dehydrogenase [13] and aldolase [14] have been found associated with the red blood cell membrane. Sigel and Pette have shown histo-

chemically that glycolytic enzymes are found along the I bands in muscle [20]. This observation stimulated studies which have shown that several glycolytic enzymes readily associate with F-actin or an F-actin · troponin · tropomyosin complex under a variety of experimental conditions [15—19]. Such evidence indicates that the glycolytic enzymes may exist as a complex or multi-enzyme system in muscle. In the present study we have examined all of the glycolytic enzymes in rat brain and have observed that several of them were associated with particulate fractions obtained from lysed nerve endings.

Materials and Experimental Methods

Brains, obtained from 1—2 month old rats of the Long-Evans strain were homogenized in 10 vols. 0.32 M sucrose with 15 strokes in a Potter-Elvehjem homogenizer fitted with a teflon pestle and centrifuged at 500 × *g*, 10 min. The resulting supernatant was centrifuged at 17 000 × *g*, 30 min, the pellet resuspended in a volume of 0.32 M sucrose equal to the homogenizing volume and layered over a discontinuous gradient of 0.8 M sucrose over 1.2 M sucrose [21]. After centrifugation at 53 000 × *g*, 2 h, the synaptosomal fraction which banded at the 0.8, 1.2 M sucrose interphase was removed and diluted 5 fold with 0.25 M sucrose and pelleted by centrifugation at 40 000 × *g*, 30 min. The nerve endings were lysed by resuspension in cold distilled water. Aliquots treated with 10 mM ATP, pH 7.0 or 100 mM NaCl and controls having no additions were centrifuged at 100 000 × *g*, 30 min. The supernatant solutions were removed and the pellet fractions were resuspended in 0.32 M sucrose. These latter fractions were immediately assayed for glycolytic enzyme activity.

The lysed synaptosomes prepared by resuspension of the pelleted synaptosomal fraction were fractionated further according to methods described by Tamir et al. [11]. The lysed nerve endings were centrifuged at 20 000 × *g*, 30 min. The pellet fraction was resuspended in 0.32 M sucrose and then placed on a gradient of 0.4, 0.6, 0.8, 1.0 and 1.2 M sucrose. The bands at the various interfaces (described as fractions A—D) and the supernatant (made up a portion of S, see below) and pellet fraction (E) were isolated following centrifugation at 53 000 × *g*, 2 h. The 20 000 × *g*, 30 min supernatant solution was centrifuged at 100 000 × *g*, 1 h and the resultant supernatant saved for assay (this fraction was combined with the supernatant from the gradient and labelled S) and the pellet resuspended in 0.32 M sucrose, placed over 0.5 M sucrose and centrifuged at 50 000 × *g*, 1 h. The fraction at the interphase (M_2A) was collected and assayed for enzyme levels and protein content. These fractions were used in the various assays.

The activities of the various enzymes assayed were carried out using previously reported assay procedures: hexokinase (EC 2.7.1.1) [22], glucosephosphate isomerase (EC 5.3.1.9) [23], phosphofructokinase (EC 2.7.1.11) [24], aldolase (EC 4.1.2.13) [8], glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12) [25], triosephosphate isomerase (EC 5.3.1.1) [26], phosphoglycerate kinase (EC 2.7.2.3) [23], phosphoglycerate phosphomutase (EC 5.4.2.1) [23], enolase (EC 4.2.1.11) [23], pyruvate kinase (EC 2.7.1.40) [11], lactate dehydrogenase (EC 1.1.1.27) [27], acetylcholinesterase (EC 3.1.1.7) [11], fumarase (EC 4.2.1.2) [28] and (Na^+ + K^+)-ATPase (total ATPase (EC 3.6.1.3) minus

Mg^{2+}-dependent ATPase (EC 3.6.1.3)) [11]. The enzymes were assayed using saturating levels of substrates. Changing the size of the aliquot to be assayed produced proportional changes in measured activity for each of the enzymes studied. Enzyme activities are expressed as μmoles of substrate catalyzed to product per minute (units) as a function of fresh weight, volume or protein concentration. All enzymes and substrates used in the coupled enzyme assays were purchased from Sigma Chemical Company, St. Louis, Mo.

Protein was determined by the method of Lowry et al. [29] after treatment for 30 min with NaOH, final concentration 1 N. The samples were diluted 20 fold before assay to prevent base interference in the assay.

Results

All of the glycolytic enzymes except hexokinase and aldolase in whole brain homogenates have been reported to be primarily in the soluble phase [3,4]. Our data, shown in Table I, support the earlier observations. Hexokinase, found to be associated with mitochondria in both whole brain preparations [2—6] and nerve ending preparations [30], and aldolase, a portion of whose activity is isolated with membrane-containing fractions [8—10] were 76% and 53% particulate in our study. The data in Table I also shows the levels of glycolytic enzymes that were soluble or sedimentable in lysed synaptosomes following centrifugation at 100 000 × *g*, 30 min. It is seen that 40—90 per cent of glucosephosphate isomerase, phosphofructokinase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were associated with the particulate fraction. Similarly, these enzymes when isolated from muscle all demonstrated considerable binding to the F-actin · tropomyosin · troponin complex also prepared from muscle homogenates [20].

Evidence that the enzymes were bound to particulate and not merely trapped in the sediment is derived from observations that ionic substances readily elute the enzymes from the particles. Treatment of lysed nerve ending suspensions with 100 mM NaCl or 10 mM ATP, pH 7.0 before centrifugation caused solubilization such that hexokinase was 33% particulate and glucosephosphate isomerase 20%, aldolase 22%, pyruvate kinase 20% and lactate dehydrogenase 15% (Table II). ATP treatment resulted in significant loss in glyceraldehyde-phosphate dehydrogenase activity perhaps because ATP caused dissociation of the enzyme into subunits [31]. Therefore, the particles were treated with NaCl which resulted in quantitative redistribution of glyceraldehyde-phosphate dehydrogenase such that the amount of activity disappearing from the particulate fraction reappeared in the soluble fraction. Similar ionic treatments have solubilized sedimentable glycolytic enzymes from muscle preparations [16,17,20] and aldolase from particulate brain preparations [8,9]. Phosphofructokinase was not solubilized by additions of sodium or potassium chloride up to concentrations of 150 mM or with ATP up to 100 mM. Triosephosphate isomerase, phosphoglycerate kinase and phosphoglycerate phosphomutase did not show significant redistribution on addition of ionic materials most likely because most of these activities were in the soluble fraction before addition of ATP or sodium chloride.

The data in Table III shows the distribution of glycolytic enzymes following

TABLE I

LEVEL OF ACTIVITY OF GLYCOLYTIC ACTIVITY IN SUPERNATANT AND PELLET FRACTIONS OF WHOLE HOMOGENATES OF BRAIN AND SYNAPTOSOMES

The data represent the activity in the supernatant solutions and the pellets after centrifugation of each fraction 100 000 × *g* 1 h. The whole homogenate of brain was first centrifuged at 500 × *g* 10 min to remove cell debris and nuclei. Preparation of synaptosomes is given in the methods section. The values reported in the table represent the units of activity obtained in each fraction and are based on 1 g fresh weight. Animals used in these experiments were 3—4 weeks of age. Each value shown represents mean ± S.D., $n = 4$.

	Units/g brain					
	Whole homogenate			Synaptosomes		
Enzyme	Supernatant	Pellet	% Soluble	Supernatant	Pellet	% Soluble
Hexokinase	2.0 ± 0.2	6.28 ± 0.1	24.1 ± 2.0	0.32 ± 0.03	2.3 ± 0.06	12.3 ± 1.4
Glucosephosphate isomerase	4.9 ± 0.1	3.7 ± 0.3	59.9 ± 7.3	0.06 ± 0.02	0.96 ± 0.11	5.9 ± 1.8
Phosphofructokinase	7.6 ± 0.1	2.53 ± 0.1	74.9 ± 1.1	0.04 ± 0.02	0.35 ± 0.03	9.4 ± 1.1
Aldolase	4.5 ± 0.1	6.3 ± 0.5	46.8 ± 10.2	0.46 ± 0.03	1.13 ± 0.19	29.6 ± 4.7
Glyceraldehyde-phosphate dehydrogenase	3.06 ± 0.2	2.02 ± 0.1	61.2 ± 4.5	0.2 ± 0.01	0.36 ± 0.06	35.8 ± 2.4
Triosephosphate isomerase	427.0 ± 38.0	70.33 ± 4.9	85.8 ± 0.4	59.7 ± 1.8	20.3 ± 0.89	74.6 ± 0.6
Phosphoglycerate kinase	48.9 ± 0.3	10.5 ± 0.5	83.0 ± 2.5	6.9 ± 0.12	1.96 ± 0.2	77.9 ± 3.4
Phosphoglycerate phosphomutase	58.9 ± 1.5	11.7 ± 0.4	83.5 ± 0.6	7.9 ± 0.13	1.24 ± 0.15	86.5 ± 2.4
Enolase	26.7 ± 0.5	4.4 ± 0.6	85.9 ± 3.0	1.7 ± 0.05	0.66 ± 0.06	72.3 ± 3.1
Pyruvate kinase	35.7 ± 1.5	19.4 ± 0.3	64.7 ± 1.9	2.3 ± 0.02	9.9 ± 0.7	19.0 ± 4.7
Lactate dehydrogenase	44.5 ± 1.1	12.8 ± 0.2	77.7 ± 0.8	5.5 ± 0.22	2.85 ± 0.26	65.9 ± 4.6

TABLE II

EFFECT OF ADDED ATP OR NaCl ON ENZYME DISTRIBUTION BETWEEN SOLUBLE AND PARTICULATE PHASES OF LYSED NERVE ENDINGS

Preparation of these fractions is described in Methods. Each value represents the mean ± S.D., $n = 3$ except glyceraldehyde-phosphate dehydrogenase where $n = 4$.

Enzyme	Fraction (units/ml)			
	—ATP		+ATP	
	Supernatant	Pellet	Supernatant	Pellet
Hexokinase	0.06 ± 0.03	0.52 ± 0.02	0.56 ± 0.01	0.27 ± 0.04
Glucosephosphate isomerase	0.02 ± 0.01	0.43 ± 0.04	0.35 ± 0.04	0.09 ± 0.03
Aldolase	0.24 ± 0.03	0.47 ± 0.07	0.56 ± 0.03	0.16 ± 0.03
Phosphoglycerate kinase	2.42 ± 0.30	0.6 ± 0.07	2.44 ± 0.60	0.43 ± 0.10
Phosphoglycerate phosphomutase	1.36 ± 0.08	0.27 ± 0.03	1.42 ± 0.07	0.30 ± 0.03
Enolase	0.74 ± 0.07	0.17 ± 0.02	0.88 ± 0.07	0.11 ± 0.10
Pyruvate kinase	0.96 ± 0.19	2.30 ± 0.32	2.75 ± 0.15	0.69 ± 0.08
Lactate dehydrogenase	1.96 ± 0.07	0.97 ± 0.17	2.68 ± 0.05	0.46 ± 0.12
Glyceraldehyde phosphate dehydrogenase	0.05 ± 0.01	0.16 ± 0.02	0.10 ± 0.05	0.02 ± 0.01
	—NaCl		+NaCl	
Glyceraldehyde-phosphate dehydrogenase	0.06 ± 0.02	0.15 ± 0.02	0.17 ± 0.02	0.02 ± 0.01
Phosphofructokinase	0.04 ± 0.02	0.23 ± 0.03	0.04 ± 0.01	0.24 ± 0.04

sub-fractionation of synaptosomal particles. The glycolytic enzymes having significant particulate activity showed the greatest specific activity in fractions reported to contain synaptosomal membranes [11,32,33]. The procedure utilized for sub-fractionation was that of Tamir et al. [11] which is a modification of procedures developed by Gray and Whittaker [32] and Rodriguez De Lores Arnaiz et al. [33]. Their data indicated that fractions enriched in synaptosomal membranes were also enriched in acetylcholinesterase and sodium potassium ATPase activities. The synaptosomal fractions, i.e. those having highest specific activities of ATPase and acetylcholinesterase are the fractions having highest specific activity of glucosephosphate isomerase, phosphofructokinase, aldolase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase. None of these enzymes parallel the specific activity pattern observed for phosphoglycerate kinase or phosphoglycerate mutase (not shown), which further suggests that the six enzymes, glucosephosphate isomerase, phosphofructokinase, aldolase, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase are uniquely associated with membrane fractions. The fractions having the highest specific activity of fumarase, an enzyme present in intact mitochondria, also had the highest

TABLE III

SPECIFIC ACTIVITY OF VARIOUS GLYCOLYTIC ENZYMES, FUMARASE, ACETYLCHOLINE ESTERASE AND SODIUM POTASSIUM ATPase IN VARIOUS SUBFRACTIONS OF LYSED NERVE ENDINGS *

Enzyme	Fraction (units/mg protein)						
	Supernatant	A	B	C	D	E	M_2A
Hexokinase	0.09 ± 0.04	0.08 ± 0.04	0.08 ± 0.05	0.21 ± 0.17	0.31 ± 0.08	0.26 ± 0.05	0.08 ± 0.06
Glucosephosphate isomerase	0.29 ± 0.04	0.9 ± 0.12	0.75 ± 0.79	0.84 ± 1.0	0.36 ± 0.35	0.23 ± 0.19	0.33 ± 0.37
Phosphofructokinase	0.04 ± 0.02	0.07 ± 0.01	0.10 ± 0.03	0.10 ± 0.04	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.02
Aldolase	0.13 ± 0.09	0.16 ± 0.12	0.09 ± 0.05	0.1 ± 0.06	0.08 ± 0.04	0.06 ± 0.05	0.1 ± 0.1
Glyceraldehyde-phosphate dehydrogenase	0.10 ± 0.03	0.17 ± 0.03	0.15 ± 0.06	0.12 ± 0.04	0.10 ± 0.04	0.03 ± 0.01	0.16 ± 0.07
Pyruvate kinase	0.67 ± 0.25	0.95 ± 0.41	1.09 ± 0.45	1.17 ± 0.73	0.92 ± 0.14	0.84 ± 0.25	0.56 ± 0.11
Lactate dehydrogenase	1.37 ± 0.39	0.88 ± 0.44	0.66 ± 0.48	0.37 ± 0.21	0.23 ± 0.09	0.20 ± 0.06	1.0 ± 0.5
Phosphoglycerate kinase	2.14 ± 0.70	0.32 ± 0.17	0.12 ± 0.06	0.11 ± 0.07	0.06 ± 0.02	0.05 ± 0.03	1.4 ± 1.4
ATPase	0.02 ± 0.02	0.21 ± 0.18	0.24 ± 0.07	0.18 ± 0.10	0.13 ± 0.08	0.08 ± 0.04	0.02 ± 0.03
Acetylcholine esterase	0.22 ± 0.16	0.88 ± 0.5	0.81 ± 0.3	0.64 ± 0.2	0.53 ± 0.1	0.3 ± 0.2	0.1 ± 0.07
Fumarase	0.60 ± 0.23	1.24 ± 0.76	1.05 ± 0.57	1.7 ± 1.7	2.44 ± 0.31	2.26 ± 1.67	0.56 ± 0.45
Protein (mg/fraction)	3.26 ± 1.06	0.8 ± 0.35	0.85 ± 0.45	0.85 ± 0.49	3.48 ± 1.25	3.93 ± 1.38	0.36 ± 0.025

* According to Rodrigues De Lores Arnaiz et al. [33] and Tamir et al. [11] supernatant represents soluble activity A, B & C are primarily synaptosomal membranes, D and E are enriched in mitochondria and M_2A is enriched in synaptic vesicles. A, B, C and D represent the fractions isolated from above the 0.8, 0.9, 1.0 and 1.2 M bands of sucrose. E represents the pelleted fraction. Following the procedure for synaptic vesicles, M_2A represents the fraction banded at the 0.32–0.5 M sucrose interphase. Supernatant represents a combination of the fraction above the 0.8 M sucrose and the 100 000 × *g* 1 h supernatant obtained while pelleting the synaptic vesicles. The system of labelling fractions is exactly that used by Tamir et al. [11]. Each value represents the mean ± S.D., $n = 4$.

specific activity of hexokinase which supports the earlier evidence that hexokinase is associated with mitochondria [30].

An earlier report implied that phosphofructokinase is associated with mitochondria [7] because approximately one third of the activity sediments at 10 000 × *g*, 10 min when using 0.3 M mannitol containing 0.5 mM EDTA as the preparatory medium. As shown in Table III, analysis of the subfractions of lysed nerve ending preparations shows enrichment of phosphofructokinase activity in fractions also enriched in ATPase and acetylcholinesterase rather than fumarase and hexokinase. Fractions obtained from initial procedures utilizing homogenates prepared in either 0.32 M sucrose or 0.3 M mannitol/0.5 mM EDTA produced identical results. These data imply that the particulate phosphofructokinase of nerve endings is associated with synaptosomal membranes rather than mitochondria.

Discussion

A few glycolytic enzymes have previously been reported to be associated with particulate fractions of rat brain homogenates [2—12]. A role for the binding of hexokinase to particles in brain has been established; it is more active than the soluble form. The K_i for glucose 6-phosphate is higher [34—37], and the K_m for ATP is lower [38—40], for the bound form than for the soluble form. Furthermore, the enzyme redistributes in vivo such that the bound or more active form predominates under conditions of energy stress [37,41] thus permitting a more rapid rate of glucose phosphorylation. The reason for other glycolytic enzymes being bound to particles in brain, or any other tissue, is unknown at the present time.

Clarke and Masters [20] recently observed that several glycolytic enzymes will associate with the F-actin · troponin · tropomyosin complex isolated from muscle tissue even under conditions of high ionic strength. In view of this observation the authors suggest it is possible that there is an interaction between glycolytic enzymes and structural proteins in muscle. Many of the glycolytic enzymes which showed a high degree of binding to actin also were found in our studies to show significant association with particulate fractions of nerve endings. The percentage of each glycolytic enzyme which bound to the F-actin complex containing 2 mM $MgCl_2$ was as follows (the percentage shown in brackets represents the particulate portion of each enzyme in synaptosomes): hexokinase 0% (88%), glucosephosphate isomerase 40% (94%), phosphofructokinase 95% (90%), aldolase 50% (70%), glyceraldehyde-phosphate dehydrogenase 45% (64%), triosephosphate isomerase 10% (25%), phosphoglycerate kinase 10% (22%), phosphoglycerate phosphomutase 5% (13%), enolase 10% (27%), pyruvate kinase 70% (81%) and lactate dehydrogenase 50% (35%). Except for hexokinase, the enzymes having a high degree of particulate activity in synaptosomes also showed considerable association with the F-actin complex. While Clarke and Masters found that hexokinase did not bind significantly to the muscle contractile proteins it was observed to be particulate in our studies presumably because of its binding to mitochondria present in nerve ending preparations [30]. As the data in Table III indicates, the enzymes showing a high degree of binding are not enriched in the mitochondrial frac-

tions. Rather, enrichment is in fractions containing the highest specific activity of acetylcholinesterase and sodium potassium ATPase implying that the association of glycolytic enzymes, under the conditions used in the present study, is with the synaptosomal membranes or some component of the membranes.

Many tissues have actin and myosin-like proteins. In brain, the actin-like protein, neurin, is associated with the synaptosomal membrane and has been postulated to be involved in the secretory mechanism at nerve endings. The results reported herein were obtained under conditions very different from those existing in vivo, therefore, whether the enzymes are associated with the synaptosomal membranes in vivo remains to be determined. It is nevertheless intriguing to speculate that if the nerve ending glycolytic enzymes were particulate they may be associated with neurin (except for hexokinase) at the synaptic membrane perhaps to provide glycolytic energy for secretion.

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