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GLYCOLYTIC ENZYME BINDING IN FETAL BRAIN - THE ROLE OF ACTIN

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The binding of aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase in fetal calf brain homogenates and extracts has been investigated at both 0° and 37°C under high ionic strength conditions. The results demonstrate far greater enzyme binding at 37°C than at 0°C, which correlates with an increased sedimentation of cytoskeletal actin at the higher temperature. A dependence of enzyme sedimentation on the presence of polymerised actin was also demonstrated, and this indicates that cytoskeletal actin is a major adsorbent of glycolytic enzymes in this non-muscle tissue.

While for a long time the enzymes of glycolysis were described as the classical soluble enzymes of the cell, it is now well established for skeletal muscle[1-3], that these enzymes are associated with the actin containing filaments of the contractile apparatus, and it has been suggested[4] that these associations lead to an organisation of the glycolytic enzymes which contributes to the regulation of glucose metabolism in this tissue. Structural proteins (actin, tropomyosin, etc.) similar to those previously established as the binding sites for glycolytic enzymes in muscle, also occur in non-muscle cells where they play a central role in the structure of the cytoskeleton and in cell motility functions[5-6]. Clearly, the potential for enzyme binding also exists in these cells and indeed there are many reports[7-9] of association between glycolytic enzymes and various particulate fractions of non-muscle cells and tissues. All of these studies however, have been carried out only under very low ionic strength conditions and so some question remains as to the physiological significance of the observed interactions. In the present report we describe the binding of

ABBREVIATIONS

ALD, aldolase; GPDH glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase; DTT, dithiothreitol; EGTA, ethyleneglycol-bis- (β-aminoethyl ether) N, N' - tetra-acetic acid.

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glycolytic enzymes in fetal calf brain homogenates and extracts under physiologically relevant conditions and provide evidence to suggest that the major adsorbent is cytoskeletal actin.

MATERIALS AND METHODS

Enzyme Binding in Homogenates: Fetal calf brains were collected from the local abattoir, transported to the laboratory and processed within 2 hours of collection. Each brain was divided down the mid line into equal portions. One half was homogenised in 3 volumes of ice cold 0.01M sodium phosphate, 0.05M KCl, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT, 0.2mMATP, pH6.8 at 0°C for 60 seconds in a Waring blender at full speed. 5ml aliquots of the homogenate were incubated at 0°C for 30 minutes then centrifuged at 40,000rpm for 60 minutes in a 50Ti rotor in a Beckman ultracentrifuge operated at 4°C. The resulting supernatant was collected and the pellet resuspended in 5ml of homogenisation buffer. The volume of both fractions was measured and samples taken for enzyme assay and determination of actin and tubulin content. The other half of the brain was processed as above except that the buffer was prewarmed to 37°C and the homogenisation and incubation carried out at 37°C before centrifugation at 25°C. Where the effect of various agents (eg. colchicine, Ca^{++}) on enzyme distribution were to be tested, they were added to the initial homogenisation buffer at the concentrations indicated.

Extract experiments: Whole brains were homogenised and centrifuged at 4°C as described above. The supernatant was collected, dialysed against 10 volumes of homogenisation buffer overnight at 4°C, then concentrated five fold using a PM-10 membrane in an Amicon stirred cell at 4°C. Total protein concentrations[10] ranged from 20-24mg/ml. 2.5ml aliquots of these concentrated extracts were incubated at either 0°C or 37°C for 30 minutes before centrifugation at 40,000rpm for 60 mins at 4°C or 25°C. The supernatants were collected and the pellets resuspended in 1ml of buffer. Samples were taken for enzyme assay, actin and tubulin determination. In other experiments 20ml of concentrated extract was incubated at 37°C and then centrifuged as described above. The resulting supernatant designated an actin-depleted extract was used in the experiment described below.

Preparation of brain actin and addition to actin-depleted brain extracts: Fetal calf brain actin was prepared using procedures similar to those previously used to obtain actin from extracts of macrophages[11] and sea urchin eggs.[12] Concentrated brain extracts were incubated at 37°C for 60 minutes following addition of MgATP and colchicine to 1mM each. A solid gel usually formed during these incubations and the gelled extract was sedimented at 40,000rpm for 60 minutes. The resulting pellet was washed twice in a modified extraction buffer which contained KCl at 0.6M, before being resuspended in normal extraction buffer. SDS gels revealed the preparation was greater than 90% actin and the presence of F-actin filaments was confirmed by electron microscopy. The preparation was devoid of glycolytic enzyme activity. To test the influence of this actin on enzyme sedimentation, 2ml aliquots of actin-depleted extracts were set up and incubated at 37°C for 30 minutes following addition of either 1ml (5mg) of brain actin or 1ml of buffer. Following centrifugation the extent of enzyme sedimentation was determined.

Enzyme assays: The glycolytic enzymes aldolase, glyceraldehyde-3-phosphate dehydrogenase (GPDH) and pyruvate kinase (PK) were measured as previously described[4] except that all assay mixtures contained 1mM rotenone to inhibit NADH oxidase activity which was found to be significant in some brain fractions. All assays were performed in triplicate at 30°C in a Cary

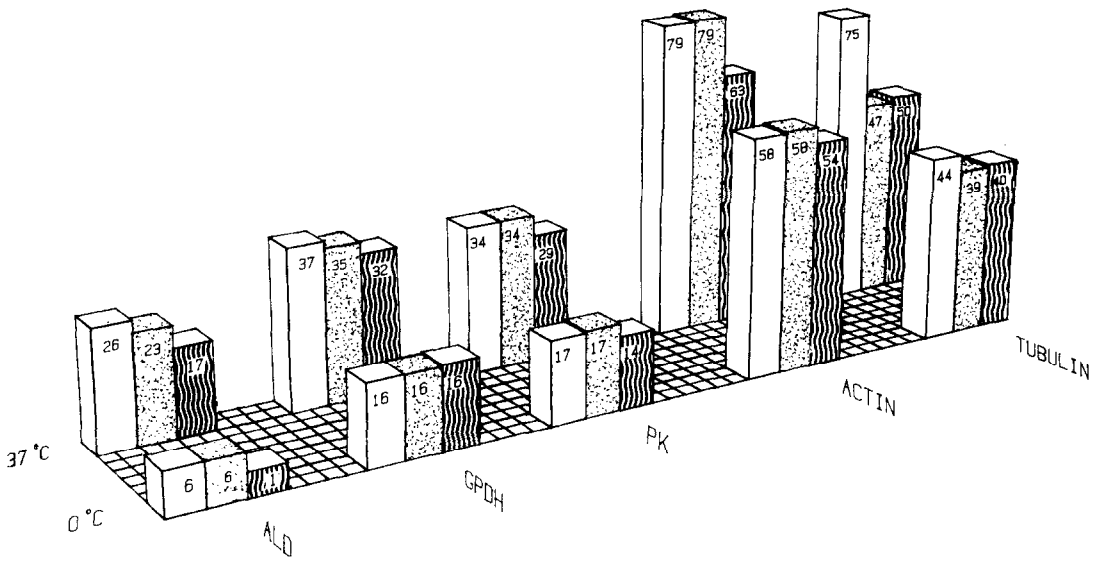

Figure 1: Enzyme and structural protein sedimentation in fetal brain homogenates at 0° and 37°C. Figures on histograms indicate % sedimentation ☐ no additions, mean of 6 experiments; values at 0° and 37°C significantly different to $p \leq 0.005$ for all parameters; ▨ in presence of 1mM colchicine, mean values of duplicate experiments; ▨ in presence of 2mM $CaCl_2$, mean values of duplicate experiments.

118 spectrophotometer on line to a Varian computer for calculation of reaction rates. The percentage enzyme sedimentation in each sample was calculated as (enzyme activity in pellet/activity in supernatant+pellet) X 100. Control experiments determined that recovery of enzyme activities ranged from 95-105%.

Actin and tubulin quantitation: The actin and tubulin content of fractions was determined by quantitative densitometry of SDS polyacrylamide gel patterns using techniques previously described[13].

RESULTS AND DISCUSSION

The distributions of the glycolytic enzymes between the soluble and particulate phases of fetal calf brain homogenates prepared at either 0°C or 37°C are compared in figure 1. At 0°C there was a small but significant sedimentation of enzymes with the particulate fraction. A large proportion of this sedimented activity was probably due to true adsorption; occlusion of enzyme within membrane bound particles such as synaptosomes is unlikely as these would be largely destroyed because of the low osmolarity of the media and the homogenisation conditions used[7]. At 37°C there was a substantial increase in the extent of enzyme association with the particulate fraction. Twice as much GPDH and PK was bound at 37°C as at 0°C while aldolase binding increased four-fold over that observed

at 0°C. The higher levels of enzyme binding are particularly noteworthy as they occurred at relatively high ionic strength. The extraction media had an ionic strength of approximately 0.08 to which must be added the tissue contribution-taken together the effective ionic strength within the homogenate was at least 0.1 - 0.12 - an ionic strength not "normally" associated with extensive glycolytic enzyme adsorption in most in vitro studies[7,8,9,17]. The distinguishing feature of the present experiments however, was the maintenance of relatively high enzyme concentrations at physiological temperature. Although the increased enzyme binding at 37°C may be multifactorial in its origin, the other data in figure 1 indicates one of the possible contributors. As shown in this diagram, increasing the temperature to 37°C led to a substantial increase in the sedimentation of the two main cytoskeletal proteins actin and tubulin, due to the increased polymerisation of these proteins which is known to occur[12,14,15] at 37°C and low Ca^{++} concentrations. The addition of the microtubule depolymerising agent, colchicine, however, reduced the extent of tubulin sedimentation but had no influence on enzyme or actin sedimentation clearly arguing against tubulin as a potential enzyme adsorbent. On the other hand, the addition of Ca^{++} which decreases the extent of actin polymerisation[11,12] probably through the actin of gelsolin [16],reduced the extent of actin and enzyme sedimentation at both temperatures (figure 1). Calcium ions also inhibit microtubule polymerisation[15], so as expected tubulin sedimentation was also decreased in the presence of this agent. Taken together these results suggest a correlation between the extent of actin polymerisation and enzyme sedimentation.

If this is so, it should also be true for concentrated extracts of brain, for in a variety of non-muscle systems[11,12] warming concentrated cell or tissue extracts in the absence of calcium ions increases polymerisation of actin. To this end then, concentrated brain extracts were prepared at 0°C and then incubated at either 0°C or 37°C before the extent of enzyme sedimentation was determined. As shown in Table I, warming of

TABLE I

		% Sedimentation			
		ALD	GPDH	PK	ACTIN
Concentrated Extract, 0°C	a	9.9±1.1	3.1±0.1	7.5±0.7	7.0±1.5
Concentrated Extract, 37°C	b	21.4±4.3	21.2±8.6	26.0±6.0	46.4±6.0
Actin-depleted Extract, 37°C	c	2.1	2.3	9.4	-
Actin-supplemented Extract, 37°C	c	13.0	15.0	15.6	-

All extracts prepared in 0.01 M sodium phosphate, 0.05 M KCl, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT, 0.2mMATP, pH6.8 as described in Methods. a, mean±SD, 3 experiments; b, mean±SD, 9 experiments; c, mean of duplicate experiments.

these concentrated extracts to 37°C led to a large increase in the extent of actin sedimentation which was accompanied by a significant increase in the extent of enzyme sedimentation. Indeed in many experiments gel formation was observed in the warmed extracts, and the formation of actin filaments and actin filament bundles was confirmed by electron microscopy. Control experiments with purified enzymes singly or as a mixture showed no more than 1-2% sedimentation of enzyme protein under similar conditions. That the increased enzyme sedimentation resulted from an association with polymerised actin is supported by the other results presented in Table I. Actin depleted extracts (see Methods) were analysed for enzyme sedimentation at 37°C with or without the addition of brain actin. As shown in Table I, little enzyme sedimentation resulted on centrifugation of the actin-depleted extracts. However on supplementation of these extracts with brain actin, further significant enzyme sedimentation occurred. When considered in conjunction with the previous results it would suggest that the increased sedimentation of glycolytic enzymes in extracts processed at 37°C was due to the increased polymerisation of actin, and that actin is the main enzyme adsorbent in these extracts.

In summary these results demonstrate that glycolytic enzymes bind significantly to actin in extracts of a non-muscle tissue under physiologically

relevant conditions of pH and ionic strength. The failure to observe such associations in the past has probably been due to the choice of experimental conditions, particularly processing tissues at low temperatures. This practice, together with lack of precautions to control the free calcium ion concentration favours extensive depolymerisation of actin, and consequently minimal enzyme sedimentation. Furthermore, most experiments have been carried out at relatively high dilutions of cellular constituents, another condition which leads to disruption of labile associations. The demonstration of actin as a major adsorbent of glycolytic enzymes in a non-muscle tissue is of some significance as the actin-containing thin filaments of the myofibril have long been known[17,2] as the main site of enzyme binding in skeletal muscle. Consequently it is proposed that this ubiquitous and highly conserved protein has a general role to play in regulating the organisation and functions of the glycolytic system in eukaryotic cells.

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