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Association of Glycolytic Enzymes with Structural Proteins

by

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DEDICATION

To my wife Debbie, whose inspiration, encouragement and patience is deeply appreciated.

To my parents, for their invaluable support throughout the course of this achievement.

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Abstract

The thesis contains data which supports the contention that glycolytic enzymes may interact with subcellular particulate structures. The structural proteins of skeletal muscle I band myofibrils may provide a base structure with which individual glycolytic enzymes may associate. Data presented in the literature review provide some evidence for enzyme interaction with structural muscle proteins. However, with the exception of aldolase, the studies did not indicate specific interactions between individual glycolytic enzymes and I band proteins. Previous attempts involved sedimentation of a mixture of enzymes with a purified structural protein preparation which provides a system for many types of protein-protein interactions. The strategy presented here involved covalent linkage of an actin or actin complex ligand to cyanogen bromide activated Sepharose 4B which served as an affinity matrix for enzymes passed through separate chromatographic columns. Enzyme activity was utilized as a marker in order to measure the degree of enzyme adsorption to the various actin columns. The ligands covalently coupled to Sepharose were comprised of purified F actin, G actin, an F-actin-tropomyosin complex cross-linked with glutaraldehyde and F actin which was also glutaraldehyde cross-linked. In an attempt to determine the degree of association of glycolytic enzymes

with the actin containing columns, individual enzymes were passed through each column at low ionic strength (0.015). A gradient of increasing ionic strength was then applied to the columns in order to release any bound enzyme. Fractions were collected and assayed. Certain glycolytic enzymes adsorbed to the various columns with different degrees of affinity. Triosephosphate isomerase, glucosephosphate isomerase, phosphoglycerate mutase, enolase and creatine phosphokinase did not adhere to the matrices. The enzymes which were found to adsorb in the columns were aldolase, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase and yeast phosphoglycerate kinase. Experiments demonstrated that glutaraldehyde cross-linked F actin provided a better ligand for enzyme interaction than did the untreated F actin. The enzymes which adsorbed provided the following profile in order of decreasing affinity; F-actin-tropomyosin > F actin glutaraldehyde cross-linked > F actin > G actin. These data support the view that the contractile proteins may serve as a site or structure for the organization and possible close association of glycolytic enzymes in the I band muscle thin filament region. This type of interaction may play an important role in the energy production in muscle cells.

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LIST OF ABBREVIATIONS

HK	hexokinase (EC. 2.7.1.1)
PHI	phosphohexose isomerase (EC. 5.3.1.9)
PGI	phosphoglucoisomerase
PFK	phosphofructokinase (EC. 2.7.1.11)
ALD	aldolase (EC. 4.1.2.b)
TPI	triosephosphate isomerase (EC. 5.3.1.1)
GAPDH	glyceraldehydephosphate dehydrogenase (EC. 1.2.1.12)
PGK	phosphoglycerate kinase (EC. 2.7.2.3)
PGM	phosphoglycerate mutase (EC. 2.7.5.3)
E	enolase (EC. 4.3.1.11)
PK	pyruvate kinase (EC. 2.7.1.40)
LDH	lactate dehydrogenase (EC. 1.1.1.27)
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CK	creatine phosphokinase (EC. 2.7.3.2)
DHAP	dihydroxyacetone phosphate
K_m	Michaelis-Menten constant
V_{max}	maximum velocity
NAD	nicotinamide adenine dinucleotide
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
SDS	sodium dodecyl sulfate
F-A	F actin
G-A	G actin
T_m	tropomyosin
F-A-Tm	F-actin-tropomyosin
Ga, Gl	glutaraldehyde
F-A-Gl	F actin glutaraldehyde cross-linked
HEPES	N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid
TRIS	TRIS (hydroxymethyl) aminomethane

Association of Glycolytic Enzymes with Structural Proteins

Introduction

The glycolytic enzymes have been traditionally viewed as residing in the cell cytoplasm as soluble unattached macromolecules. This concept was derived from the fact that these enzymes could be easily extracted in dilute aqueous solutions from disrupted cells. The question of compartmentation of these enzymes within a microenvironment has attracted a great deal of interest in recent years (1,2). There are several structural compartments in eukaryotic cells involved with the various phases of carbohydrate metabolism. The Krebs cycle enzymes are considered to be mitochondrial in location; enzymes of glycogen metabolism are complexed to glycogen particles (3), while the enzymes involved in the pentose phosphate shunt appear to be microsomal (4). Within these separate cytosolic compartments the possibility of a discrete organization of glycolytic enzymes exists. The cytoplasm *in vivo* is gel like and composed of a large number of organelles with multiple compartments separated and connected by a variety of different membranes and particulate structures such as microfilaments and microtubules. The fact that the cellular milieu consists of a highly concentrated protein solution with structural barriers suggests a possibility for definite organization

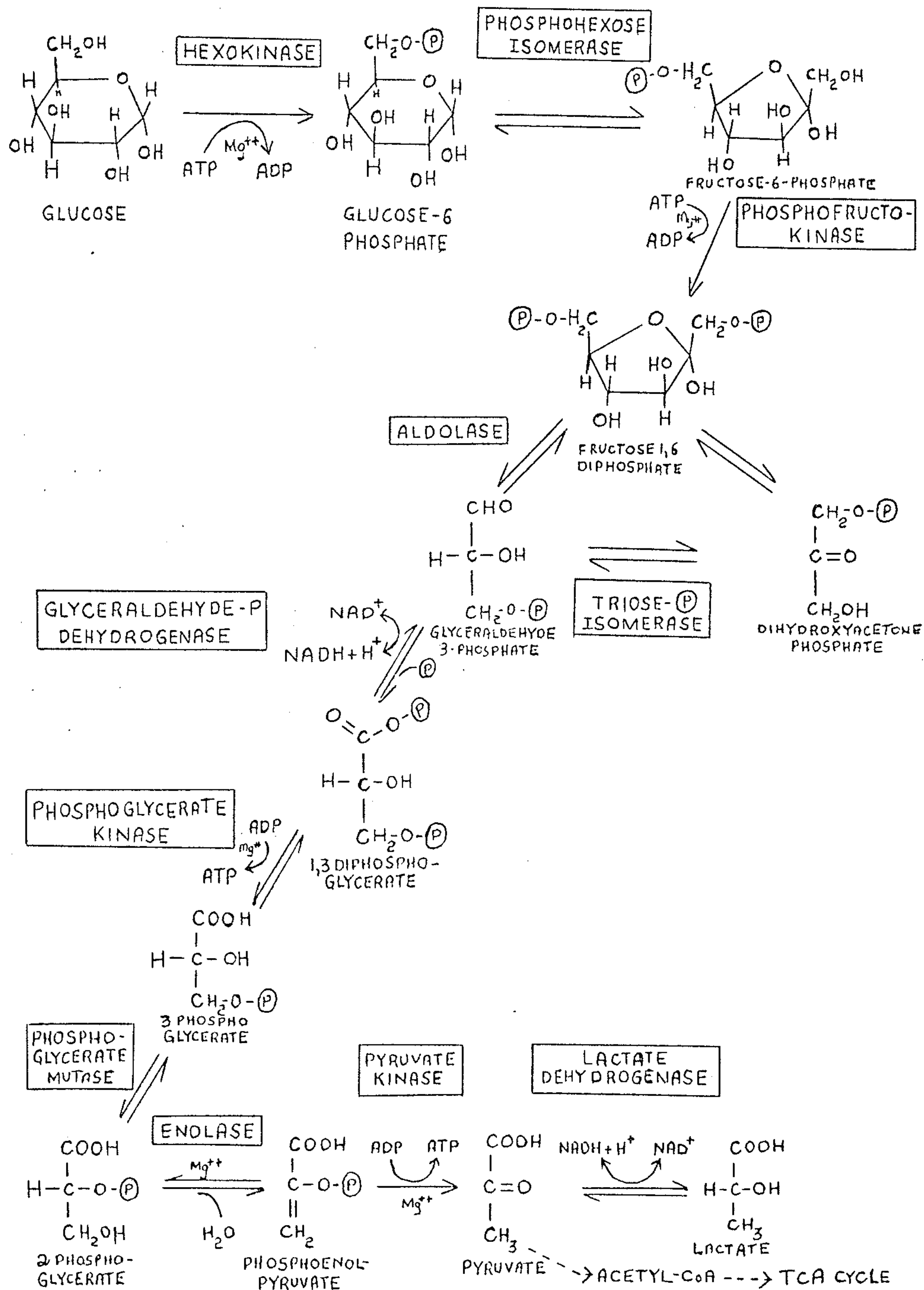
of the glycolytic enzymes rather than a disoriented random arrangement. It is important to note that the disruption of cellular structures and the dilution of the cytoplasm during tissue homogenization and fractionation produces a medium of lower protein concentration that can not be equated to the cellular environment _in vivo_. Evidence is accumulating that indicates glycolytic enzymes do associate with various subcellular components. In particular, certain glycolytic enzymes appear to reversibly interact with varying degrees of specificity to the skeletal muscle thin filament structural proteins of the I band; F actin, tropomyosin and troponin.

Metabolism of D-glucose for production of energy can be accomplished in two ways. Glycolysis (Embden-Meyerhof pathway) takes place in the absence or presence of oxygen and involves the breakdown of glucose into two pyruvate molecules. Under anaerobic conditions pyruvate is converted to lactate. Under aerobic conditions, pyruvate is oxidized in the mitochondria by way of the Krebs cycle. The glycolytic pathway involves a series of eleven reactions each catalyzed by a particular enzyme. A consideration of metabolic control reveals that certain key enzymes regulate the process. Notably, hexokinase, phosphofructokinase and pyruvate kinase activities can be influenced by interactions with metabolites which can activate or inhibit enzyme activity. The HK reaction is regulated by the levels

of glucose-6-phosphate, adenine nucleotides and Pi (5). PFK is influenced by ATP, ADP, AMP, fructose diphosphate, fructose-6-phosphate, citrate and phosphate (6) while PK is altered by phosphoenolpyuvate, fructose diphosphate, ATP, creatine phosphate and cations such as Mg^{2+}, K^{+} and NH_4^{+} (7). Even though all these factors are believed to regulate the rate of glucose metabolism, further control features are needed to account for the observed regulation of glucose catabolism (8). In view of the physiological reality of the cell's interior, the possibility of interaction of glycolytic enzymes with subcellular particles may provide alternate means of influencing enzyme catalysis and the regulation of the metabolic process as a whole. Adsorption of glycolytic enzymes to subcellular particles such as the thin filament proteins may increase the concentration of the enzymes at specific sites allowing for increased efficiency in glycolytic energy production.

Fig. 1. Diagrammatic representation of the glycolytic pathway.

GLYCOLYSIS



Theory

The classical concept of glycolytic enzymes being homogeneously distributed throughout the cytoplasm requires reconsideration in view of recent reports. A large number of studies now support the realistic contention of glycolytic enzymes organizing by means of weakly interacting forces or perhaps in association with stable subcellular components. The purpose of this study is intended to illustrate the degree of glycolytic enzyme interaction with structural proteins of the skeletal muscle I band.

The majority of studies in this area involved ultracentrifugation of enzyme mixtures from muscle homogenates with purified actin and its associated contractile proteins (refer to pp 8, literature review). Duplication of the ultracentrifugation studies of Clarke and Masters (9) was initially attempted. F actin was suspended with a muscle myogen preparation (defined pp 10) and pelleted at forces greater than 100,000 x g. The data obtained from several experiments were not acceptable due to difficulty found in the quantitation of glycolytic enzymes in the supernatant and F actin pellet. A typical study is shown in Table 1. A total decrease in enzyme activity was consistently found in samples assayed after centrifugation. The results, however, did suggest

TABLE 1. EFFECT OF IONIC STRENGTH ON THE ADSORPTION OF GLYCOLYTIC ENZYMES TO F ACTIN

The mixtures contained 9.0 mg myogen and 2.0 mg F actin in a final volume of 2.5 ml of 0.005 M imidazole, 0.001 M $MgCl_2$, 2.0 mM β-mercaptoethanol, pH=6.8 which included 5.0 mM or 150 mM KCl.

SAMPLE	FRACTION	LDH	PK	ALD	GK	PHI	TPI
muscle	S	1.66	2.90	0.59	1.54	0.28	6.18
myogen	P	0.59	0.53	0.03	0.01	0.03	0.52
myogen	S	1.43	3.11	0.28	1.47	0.33	6.13
+ actin (5 mM KCl)	P	0.32	0.90	0.25	0.23	0.06	0.88
myogen	S	1.26	2.94	0.69	1.22	0.29	5.96
+ actin (150 mM KCl)	P	0.51	0.98	0.10	0.34	0.11	0.97

association of glycolytic enzymes with actin.

This scheme did not allow for differentiation of enzyme-enzyme interaction and enzyme-actin interaction. The techniques employed to identify the possible existence of enzyme interaction involved devising a biospecific affinity system between actin and individual glycolytic enzymes.

Model

The model described herein represents a means of illustrating specific interactions between glycolytic enzymes and thin filament structural proteins. F actin, G actin and F-actin-tropomyosin are each covalently immobilized to affinity support columns. Each of the ligands serves as a receptor to which individual glycolytic enzymes may associate. Enzymes are passed through the separate columns and the columns are washed with additional buffer in order to facilitate release of unbound enzymes. Enzymes that are bound to the column are then released by application of a continuous gradient of increasing ionic strength. The advantages of such a technique include: 1) quantitation of enzyme bound to each column, 2) investigation as to the nature of interaction, eg. electrostatic, hydrophobic, 3) indication of the relative binding affinities of each enzyme, 4) determination of the relative amounts of each enzyme in association with actin

and associated proteins, and, 5) study of enzyme-enzyme interactions as a consequence of binding of one enzyme to actin. Results of these studies may provide credence to the existence of a localized complex of glycolytic enzymes within the muscle I band *in vivo*.

LITERATURE REVIEW

A review of the literature supports the contention that glycolytic enzymes may interact with various types of subcellular components or interact with each other. Interaction has been observed in many cell types ranging from unicellular organisms to mammalian tissue cells.

Evidence for the Existence of a Glycolytic Complex or Particle

An active particle bound lactate dehydrogenase (LDH) sediments in homogenates of the ciliated protozoa Tetrahymena pyriformus (10). The LDH is retained by the particles even after detergent treatment suggesting that the enzyme is firmly bound in the cell and not an artifact of fractionation procedures. A later study established the localization of other glycolytic enzymes in this organism (11). At pH 6.8 most of the hexokinase (HK) activity occurred in the 100,000 x g supernatant fraction while at pH 7.9 most of the HK activity was bound to a mitochondrial fraction (ie. binding was dependent on pH).

A number of workers have searched for the existence of a "multienzyme glycolytic complex or particle". In the unicellular organism trypanasome, a stable microbody-like

organelle containing all the glycolytic enzymes in the sequence HK to phosphoglycerate kinase (PGK) and glycerol kinase {except for triosephosphate isomerase (TPI)} has been identified and termed the "glycosome" (12). The search for such a complex has been attempted in other cells . Recent data indicates that several glycolytic enzymes from E. coli K-12 can self aggregate (13,14). Application of this fraction to a gel chromatography column gave an elution pattern resembling an aggregate of high molecular weight. Electron microscopic pictures showed a specific aggregate. The fraction contained all of the glycolytic enzymes in equimolar proportions and the catalytic ability of the particle could be altered by a decrease in the protein concentration in the concentrated enzyme fraction.

It was also discovered that glycolytic enzymes present in many tissues are found in equimolar amounts. Pette et al were the first workers to show the activities of five enzymes-TPI, glyceraldehydephosphate dehydrogenase (GAPDH), PGK, phosphoglycerate mutase (PGM), and enolase (E) had a constant ratio in muscle types from different species (15,16,17). They postulated that these enzymes, referred to as the phosphotriose-glycerate (PTG) group or constant proportion group, were organized into a constant proportion multi-enzyme complex. Attempts to identify such a complex included application of the techniques used in gel filtration, DEAE ion exhange chromatography,

ultracentrifugation and kinetic analysis. Other data did not support the hypothesis of the existence of a constant proportion complex. No evidence was obtained that this group existed as a multi-enzyme complex in homogenates of chicken breast muscle (18). Deduve was unsuccessful in locating a glycolytic complex in liver cytoplasm (19). However, a specific aggregate of glycolytic enzymes in a skeletal muscle myogen preparation, a muscle extract to containing mostly glycolytic enzymes (at high u) , was shown to form a single slow moving peak during boundary electrophoresis, compatible with the behavior of a complex (20). In another study, utilizing the same technique, complexes were documented where several _in vivo_ conditions were approximated ie. _in vivo_ ionic strength, pH, protein concentration, myosin concentration and delta protein (a tropomyosin polymer) (21). It was also observed that several glycolytic enzymes in a myogen preparation co-sedimented (22). This association was aided by the presence of myosin.

Other data has indicated that glycolytic enzymes may associate or interact with each other. Foldi (23) examined the possibility of aldolase (ALD) binding to some other component in a concentrated rabbit muscle extract (myogen) using frontal analysis gel filtration. Myogen was applied at 20° C to a Sephadex G-200 column equilibrated with buffer. It was found that the pure ALD eluted from the

column as expected on the basis of molecular weight, however ALD in the myogen preparation had an elution profile displaced towards a higher molecular weight indicating a complex formation with other macromolecules in the extract.

Sedimentation studies on a rat muscle cytoplasmic fraction provided further evidence of a multienzyme aggregate. A dialysed myogen preparation (24) approximating physiological conditions was centrifuged at high speeds along with control samples containing commercially purified enzymes (25). It was found that LDH, PK, ALD, and PFK sedimented at a much faster rate in the myogen than expected for the individual enzymes. It was also noted that this complex was very sensitive to factors such as pH, ionic strength, metabolites and the concentration of protein. Other tests for macromolecular association have been done by rapid kinetic analysis. An interaction was found between purified rabbit muscle ALD and GAPDH. Kinetic analysis indicated the reactions were more rapid than a random collision mechanism based on independent assay of each enzyme involved in the consecutive reactions catalyzed by this coupled enzyme sequence (26). It was also noted that ALD activity in myogen was augmented when GAPDH was added (27). These data suggest that enzymatic activities of adjacent metabolic sequence enzymes may be enhanced or

reduced by each other as a mechanism in which metabolic rates are regulated *in vivo*.

The existence of a multi-glycolytic enzyme complex has been postulated by many investigators. The proof for such a complex is lacking, which is probably attributable to the lack of well-researched examples of weak protein-protein interactions in the literature. Even though tightly bound multienzyme complexes are evident such as the pyruvate dehydrogenase complex and weaker interacting complexes have been found like the lactose synthetase and tryptophan synthetase complexes, weakly interacting associations been difficult to demonstrate, ie. glycolytic enzyme-membrane, enzyme-enzyme association.

There is however growing evidence that glycolytic enzymes may interact with a variety of subcellular components and particulate structures in different cell types.

Let us first examine recent work on the previously termed "soluble" (although not a glycolytic enzyme) enzyme creatine kinase (CK). The "M band" of muscle found in the middle of the A band region can be extracted with a dilute salt solution (u=0.05). A major component of this M band is creatine kinase (28,29,30). This type of evidence indicates that it is possible for an enzyme thought to be soluble to have a structural location. An important fraction of the total CK in the tissue is an integral part of the M line

while the rest is soluble or located between the inner and outer mitochondrial membrane. An antibody produced against chicken skeletal muscle CK bound specifically to the M line of the myofibrils (31). Furthermore, the incubation of monovalent F_{ab} fragments with myofibril bound CK solubilized most of the bound CK. The explanation given for the solubilization is that of a conformational change which occurs following CK interaction with F_{ab}. Thus CK no longer binds with the M line binding sites. These data support the view that CK is a physical part of the M line. Clearly it can be noted that there are forces which can bind an enzyme to a cytoplasmic structure in an intact cell even though in vitro low ionic strength (less than u=0.015) solubilizes the complex!

Mitochondria

The interaction of mammalian hexokinase with mitochondria has been documented. Since the observation by Crane and Sols (32) that HK can be found in a soluble and particulate bound form in many tissues, much work has been done involving the nature of the association with mitochondria and the physiological significance of the two forms of HK. A large fraction of the total HK activity is associated with mitochondria in chicken and rat skeletal muscle (33,34), intestinal tissue (34,35,36,37), heart

(38,39) and brain (5,40,41,42,43). Johnson (44) fractionated rat brain homogenates and found that eighty percent or more of the HK was particle bound while the majority of the other enzymes were found mainly in the supernatant fraction. This was an interesting observation since HK is known to be important in controlling glycolysis in brain (8,45). Certain metabolites and inorganic ions were able to cause solubilization of bound HK. These findings led to investigations of HK involvement in the regulation of glycolysis. Contradictory results have been reported. Kosow and Rose (47) examined ascites tumor cells under a variety of conditions but did not find enzyme redistribution. In contrast to their findings, physiological concentrations of glucose-6-phosphate or ATP specifically induced the release of ascites tumor bound HK which was first described by Rose and Warms (46). A number of factors affected the soluble-particulate distribution of HK activity. A high concentration of NaCl (0.3 mM) and ATP (1.6 mM) released ninety percent of the HK activity. Glucose-6-phosphate specifically eluted HK from ascites tumor mitochondria while inorganic phosphate or Mg^{2+} inhibited the elution. The capacity of mitochondria to bind HK was also evaluated. The amount of HK adsorbed varied with the treatment of the enzyme, eg. chymotrypsin caused loss of the capacity to rebind.

Sucrose extracts (0.3 M) of minced pig heart muscle