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Review Letter

Where is the glycolytic complex?

A critical evaluation of present data from muscle tissue

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Associations between glycolytic enzymes and subcellular structures have been interpreted as presenting a novel mechanism of glycolytic control reversible enzyme binding to subcellular structural components is believed to regulate enzyme activity in vivo through the formation of a multi enzyme complex However, three lines of evidence suggest that enzyme binding to cellular structures is not involved in the control of glycolysis (i) Calculations of the distribution of glycolytic enzymes under the physiological cellular conditions of higher ionic strength and higher enzyme concentrations indicate that a large multi-enzyme complex would not exist (ii) In many cases, binding to subcellular structures is accompanied by changes in enzyme kinetic parameters brought about by allosteric modification, but these changes often inhibit enzyme activity (iii) In the case where formation of binary enzyme enzyme complexes activates enzymes the overall increase in flux through the enzyme reaction is negligible

Enzyme binding, Mathematical modelling, Skeletal muscle, Glycolytic enzyme complexes, Glycolytic control

1 INTRODUCTION

The existence of a functional glycolytic complex in muscle cytoplasm, capable of completely metabolizing fructose 6-phosphate to pyruvic acid, is still an unsettled issue [1–3]. To date, several different methodologies have been employed to estimate the degree of enzyme association in vivo The aim of these studies was to correlate changes in enzyme binding with changes in flux through specific enzyme loci. These studies included: (i) centrifugation of tissue homogenates prepared in buffers of low ionic strength in an attempt to isolate stable enzyme complexes (homogenization experiments) [4–6]; (ii) in vitro kinetic and binding studies with purified components to demonstrate the possible existence of specific complexes [7,8]; and (iii) mathematical modelling of the proposed complex to extrapolate in vitro results to in vivo conditions [9] Problems inherent in the first two types of studies have rendered data interpretation difficult as discussed below In this case, then, mathematical modelling can provide insights into the in vivo bound and free distribution as well as the kinetic ramifications of enzyme binding.

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2. THE NON-SPECIFIC NATURE OF ENZYME BINDING

Initial studies of enzyme binding to subcellular structures involved homogenization of muscle tissue in isosmotic sucrose of low ionic strength followed by separation of bound and free fractions by centrifugation at $10\,000 \times g$. This procedure was known to sediment myofibrillar proteins such as F-actin, myosin, troponin and tropomyosin as well as subcellular organelles [10] Because enzyme precipitation increased as a result of exercise in skeletal muscle [4,6] ischaemia in ventricle [5], or anoxia in whelk foot muscle [11], this suggested that a glycolytic complex formed as a response to increased metabolic demand for ATP. The results from these experiments supplemented those from in vitro experiments that showed that glycolytic enzymes bound to F-actin under conditions of low ionic strength [8]. Studies such as these led researchers to conclude that F-actin was the cellular site of glycolytic enzyme binding in vivo.

It is not surprising, however, that glycolytic enzymes bind F-actin when tissues are homogenized at low ionic strength. This binding results from increased electrostatic interactions between positively charged proteins and negatively charged actomyosin polymers (Table I). The highly ionic nature of the enzyme/particulate matter interaction, as well as the non-specific nature of this interaction, is demonstrated by reduced enzyme binding in the presence of: (i) increasing salt concentra-

Table I

Isoelectric points of glycolytic enzymes, actin and myosin

Protein	pI	Source
PFK	5.0	pig liver
ALD	8.4	rabbit muscle
TPI	6.8	rabbit muscle
GAPDH	8.5	rabbit muscle
PGK	6.4	cow liver
PGM	9.3	sheep muscle
Enolase	8.6	rabbit muscle
PK	8.2	rabbit muscle
LDH	8.5	rabbit muscle
Actin	5.0	rabbit muscle
Myosin	5.8–6.2	chicken muscle

Values were taken from Malamud and Drysdale [34] and Deham [35]

Table II

Percentage and activity of glycolytic enzymes associated with F-actin

Enzyme	Bound conc. (mmol/kg muscle)	% of total conc. associated with F-actin	% of total activity associated with F-actin
PFK	0.29	24	49
aldolase	1.16	1	0
TPI	1.67	8	8
GAPDH	5.11	3	3
PGK	0.012	0.05	0.05
PK	4.28	50	28
LDH	0.31	2	0.02

Concentrations of bound species were determined using in vitro determined binding constants as described in the text. Enzyme activities were calculated by multiplying the relative activity of each enzyme species by its concentration and specific activity (see [9])

tions (to approximately 100 mM) [2,10,12]; (ii) increasing metabolite concentrations (to 5–10 times bound enzyme K_m values) [10,13], and (iii) increasing pH [4,14]. These conclusions are supported by experiments designed to more accurately measure enzyme binding under the higher salt and protein concentrations that exist in vivo. These latter studies showed that only a small percentage of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and pyruvate kinase (PK) were associated with particulate matter when measured using a rapid-pressure procedure (which did not dilute the cellular milieu) even though significant portions of these enzymes were bound when measured by the homogenization method [2].

Mathematical modelling of the F-actin/glycolytic enzyme interaction allowed an assessment of the relative effects of high (physiological) ionic strength and protein concentrations. Studies of this type generally support the conclusions of the homogenization studies, a relatively small percentage of TPI, GAPDH, PGK, and LDH would be associated with F-actin under cellular conditions (see Table II). On the other hand, the data of Table II also show that a large proportion of PFK and PK would be bound to F-actin. The high percentage of total PFK bound to F-actin agrees with results from a rapid-pressure procedure [2] which found that about 30% of the total PFK was associated with particulate matter, lending further support to these calculations.

Despite the relatively large proportion of total PFK and PK bound to F-actin, the data of Table II also suggest that a *complete* glycolytic complex would not exist. Firstly, it is apparent that a glycolytic complex, composed of equimolar amounts (or defined proportions [15]) of each glycolytic enzyme does not exist on F-actin. And secondly, both aldolase and PGK activities would be effectively absent from any glycolytic complex on F-actin. The absence of bound aldolase and PGK shows that the glycolytic complex would be incomplete, lacking the ability to catalyse the conversion of fructose 1,6-bisphosphate and 1,3-diphosphoglycerate to their respective products.

3 ANOMALOUS KINETICS OF BOUND ENZYMES

Kinetic studies of bound enzyme/F-actin complexes have shown diverse results. For example, F-actin bound PFK was activated when compared with the soluble enzyme [16–19] because of a relatively lower K_m for fructose 6-phosphate and a relatively higher I_{50} value for Mg's-ATP [18,19]. On the other hand, aldolase [20], GAPDH [12], PK [21], and LDH [13], were all inactivated when bound to F-actin. In the case of aldolase, F-actin competitively inhibited fructose 1,6-bisphosphate binding [22]. F-Actin associated LDH, PK, and GAPDH also have lower substrate affinities (higher K_m values) than their soluble counterparts.

The activation of bound PFK, and the inhibition of bound aldolase, GAPDH, PK, and LDH argues strongly against the formation of a functional glycolytic complex on F-actin polymers. If enzyme binding is a mechanism for increasing glycolytic flux (as suggested by the positive correlation between increased enzyme binding and exercise [4–6]) all enzymes in the purported complex should show a coordinated behaviour. In this case, we predict that all the enzymes should show increased activity when associated with F-actin [4–6]. In fact, aldolase is completely inactive when bound, and must be solubilized so that substrate can bind to its active site [22]. This demonstrates that these enzymes cannot be part of any functional glycolytic complex associated with F-actin and suggests that a bound glycolytic complex does not exist in muscle.

4. SOLUBLE COMPLEXES

A large amount of recent evidence suggests that some proteins can reversibly associate to form stable, binary complexes at relatively high ionic strengths in vitro. For

Table III

Effect of complex formation on PFK total activity determined by mathematical modelling of binding interactions

Enzyme complexes	Concentration (nmol g muscle) of PFK species		
	PFK alone	PFK plus aldolase and FBPase	PFK plus aldolase, FBPase and F-actin
PFK_1	0.7689	0.1347	0.1493
PFK_2	0.8155	0.0250	0.0308
PFK_2-Ald_4		2.2147	2.0438
PFK_4-FBPase	-	0.0003	0.0004
PFK_4-F-actin		-	0.0722
Maximal relative activity	1.00	0.64	0.72

Concentrations were calculated according to [11] using dissociation constants determined from in vitro binding experiments. Relative activity was measured by multiplying the concentration of each complex by its relative maximal velocity. Overall relative activity is the sum of the individual relative activities of each species. Total initial protein concentrations are PFK (1.2 nmol/g muscle), aldolase (40.6 nmol/g muscle), FBPase (0.4 nmol/g muscle)[16], and F-actin (14 nmol/g muscle) [22]. Dissociation constants were obtained from [33] and [19].

example, the following complexes have been observed in vitro. PFK-aldolase [23], PFK-fructose 1,6-biphosphatase (FBPase) [24], aldolase-triose phosphate isomerase (TPI) [25], aldolase-GAPDH [26], aldolase-α-glycerol-phosphate dehydrogenase (αGPDH) [27], and GAPDH-α-GPDH [27]. However, in the majority of these binding experiments, the proteins were derivatized using fluorescein isothiocyanate (FITC) which has been shown to promote enzyme associations. The effect of FITC was dramatically demonstrated by Masters and Winzor [28] who observed no interaction between aldolase and GAPDH when native (non-derivatized) proteins were used. Derivatizing protein with large, hydrocarbon ring structures may increase protein hydrophobicity and lead to increased interactions between other enzyme pairs studied in this manner. The stoichiometries of several of the complexes indicate that these complexes are (at least) partially held together by hydrophobic forces: PFK dimers interact with either aldolase or FBPase, and GAPDH dimers interact with aldolase.

Analysis of enzyme activity in soluble complexes also showed a varied response: enzymes either showed no change, were activated, or were inhibited when bound to other glycolytic enzymes. For example, no change in activity was observed for either aldolase or GAPDH when bound together in a complex [29,30]. A relative increase in αGPDH activity was observed in aldolase/αGPDH complexes (aldolase shifted the αGPDH monomer/dimer equilibrium to the active dimer form [31]). PFK activity was also increased when bound to either aldolase [23] or FBPase [32]. A decreased overall rate of glyceraldehyde 3-phosphate formation was observed when TPI was added to an aldolase-GAPDH complex [25], and a decrease in aldolase activity was observed when bound to GAPDH [29]. Analogous to the arguments against the formation of a complete glycolytic complex on F-actin, the anomalous kinetic response of individual complexes suggests that a large multi-enzyme soluble glycolytic complex also would not exist in muscle cytoplasm. If a single complex existed and metabolized glucose at a faster rate than that of the free enzymes, one would except that all enzymes in the complex would be at least as active as their free counterparts.

How important are individual enzyme/enzyme complexes in regulating overall glycolytic flux? It is possible to answer this question by considering overall PFK activity in free and complexed forms since PFK is usually considered to be the rate controlling step of glycolysis. The values of Table III compare the total activity of a solution of PFK to that of PFK in the presence of aldolase and FBPase, and to that of PFK in the presence of aldolase, FBPase and F-actin. Tetrameric PFK (PFK_4) dissociates into the catalytically inactive dimeric PFK (PFK_2). But in the presence of either aldolase, FBPase or F-actin, the overall PFK activity is increased via (i) PFK_2 binding to aldolase to partially activate the dimer [33], and (ii) PFK_4 binding to FBPase [32] and F-actin [17–19] to allosterically activate the enzyme by decreasing the K_m for substrate and increasing the I_{50} for inhibitor. Mathematical modelling of the enzyme/subcellular structure systems of Table III revealed that, even though aldolase activated inactive PFK_2, overall PFK activity was decreased in the presence of aldolase. This occurred because the high physiological concentration of aldolase drives the formation of the less catalytically active PFK_2-aldolase complex [9]; the net effect of aldolase binding would be to inhibit overall PFK activity in vivo. These calculations show that, although significant kinetic advantages may be associated with enzyme binding, these do not always translate into an increase in overall flux through specific enzyme loci. This argues against any significant contribution of complexes between PFK and glycolytic enzymes as a mechanism of increasing metabolic flux.

5. WHAT IS THE FUNCTIONAL SIGNIFICANCE OF ENZYME BINDING?

The combined weight of the in vitro and in vivo studies of enzyme/enzyme and enzyme/F-actin interactions at low ionic strength indicate that some glycolytic enzymes are more ubiquitous than others. In fact, the mathematical calculations of Tables II and III show that a significant proportion of some glycolytic enzymes may be associated in complexes in vivo [9]. However, the non-specific nature of enzyme/F-actin binding, and the anomalous kinetic responses to enzyme binding suggest that a functional multi-enzyme

glycolytic complex does not exist in vivo in muscle tissue.

What is the physiological significance of individual binary enzyme complexes, or enzyme/F-actin complexes? A possible explanation for PFK complex formation comes from mathematical modelling of PFK activity in a quaternary protein system containing PFK, F-actin, aldolase, and FBPase. In this system, binding of PFK to F-actin resulted in an overall increase in flux through PFK. This net activation resulted partly from a shift in the concentration of PFK species induced by binding to F-actin (the concentration of the PFK_2/aldolase complex decreased and the concentration of the more active PFK_4 species increased) and partly from an allosteric activation of PFK_1 when bound to F-actin [19]. These calculations, then, suggest that reversible association of individual enzyme complexes may increase the overall activity of specific enzymes without the need to form larger, multi-enzyme complexes. In this case, formation of a binary enzyme complex decreased the overall enzyme rate, whereas formation of a single enzyme/structural protein complex increased activity. Similar reversible complex formation may also serve to activate or inhibit other glycolytic enzymes and may serve as possible novel enzyme control mechanisms.

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