Specific characteristics of phosphofructokinase-microtubule interaction

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Abstract Muscle phosphofructokinase interacts with microtubule-associated protein-free microtubules resulting in a reduction of the overall activity of the enzyme [Lehotzky et al. (1993) J. Biol. Chem. 268, 10888–10894] and periodical cross-linking of the tubules [Lehotzky et al. (1994) Biochem. Biophys. Res. Commun. 204, 585–591]. Microtubule polymers of 'tail-free' tubulin obtained by removal of the carboxy-termini with limited subtilisin digestion retain the binding domains for phosphofructokinase that cross-bridges microtubule 'bodies'. Microtubule-associated proteins bound on tubulin 'tails' do not perturb the kinase binding. These data suggest that the tubulin carboxy-terminal domain is not involved in microtubule-phosphofructokinase interactions and phosphofructokinase and microtubule-associated proteins have distinct binding domains on microtubules. Of different isoforms of phosphofructokinase, occurring mainly in brain and tumor cells, the muscle isoform exhibits selective adsorption behaviour on microtubules. Phosphofructokinase M and C isoforms with different associative and allosteric properties may represent an auxiliary pathway to modulate energy production via glycolysis.

Key words: Phosphofructokinase; Microtubule; Microtubule-associated protein (MAP); Phosphofructokinase isoform; Proteolysis; Binding domain

1. Introduction

Microtubules (MTs), polymers of tubulin heterodimers, are ubiquitous cellular structures involved in several essential functions of the cell, including modulation of cell shape, intracellular transport of organelles, motility, secretion and cell division [1]. The tubulin heterodimer is composed of α and β subunits, each possesses an amino-terminal domain and a carboxy-terminal domain whose extreme end is highly charged. Limited subtilisin treatment cleaves the small carboxy-terminal fragments (molecular mass <2 kDa) from both subunits, resulting in a derivative of substantially altered assembly properties [2]. The cleaved, less acidic protein retains its competence to polymerize with a critical concentration for assembly about 50-fold lower than that for intact tubulin. It was proposed that carboxy-termini of the subunits normally impede polymerization [2]. Electron microscopy of negatively stained samples showed [2] that the cleaved protein forms ordered array of filaments which are different from MTs in that they are not closed and show clear cross-striations.

The stability and spatial arrangement of the tubules are regulated by microtubule-associated proteins (MAPs), predominanly MAP-2 and tau which contain common sequences in tubulin binding sites [3], and interact with the C-terminal domain of tubulin [4] that is exposed on the surface of tubules.

Extensive binding of many, but not all glycolytic enzymes purified from muscle to the tubulin/MTs system has been documented [5,6,7]. In addition, association of several glycolytic enzymes from brain homogenate or synaptosomes has been reported, however, only phosphofructokinase (PFK) showed considerable association even at physiological salt concentration. In in vivo experiments PFK has been identified in axonal transport as slow component b (SCb) with several other enzymes of intermediary metabolism which are organized as a discrete macromolecular cellular entity [8,9]. Associations of glyceraldehyde phosphate dehydrogenase and PFK with MAP-free MTs appear to have 'functional duality' in which the overall activities of the enzymes are modulated as well as the ultrastructure of MTs is altered [10,11,12,13,14]. Electron microscopic studies provided direct evidence for the periodical cross-bridges of MTs by PFK [14].

Recent data showed that the acidic C-terminal 'tail' of tubulin was predominantly involved in the binding of aldolase [15,16,17] and probably glyceraldehyde phosphate dehydrogenase [16]. The removal of the carboxy-terminal of tubulin heterodimer molecules, organized in MTs, with limited subtilisin digestion decreased significantly aldolase binding and aldolase inhibited subtilisin-catalyzed cleavage of the C-terminal [15]. The C-terminal 'tail' of both tubulin subunits is rich in acidic amino acids, thus it is highly charged at physiological pH and exposed in an extended conformation. Most glycolytic enzymes are 'basic' proteins with isoelectric points around 8 [18] and electrostatic forces are predominantly involved in the heterologous interactions. PFK is an exception concerning its relatively low isoelectric point that questions the role of the acidic tubulin 'tail' in the binding of PFK.

All these data refer to the muscle isoform of PFK whereas in certain vertebrate tissues the kinase and some other glycolytic enzymes exist as series of multiple forms. The brain possesses a complex mixture of homotetramers and hybrids [19]. Three isoforms of PFK exist: C (brain type), M (muscle type) and L (liver type) [20] that are easily resolvable by SDS-PAGE. In normal adult brain, the M–C hybrids of PFK are predominant with a small amount of M–L. The C isoform, found in relatively high ratio in tumor cells, possesses altered allosteric properties in comparison to the muscle-type M isoform [20,21]. The hybrids may produce properties which are intermediate or similar to those found for homotetramers.

In this study we provide evidence against the direct involvement of the tubulin carboxy-terminal 'tails' as well as that of the MAPs in PFK binding to MTs. Differential binding behaviour of the brain PFK isoforms is demonstrated as well.

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Abbreviations: PFK, phosphofructokinase; MT, microtubule; MAP, microtubule-associated protein.

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2. Materials and methods

Rabbit muscle PFK, GTP, phenylmethylsulfonyl fluoride and taxol were from Sigma, other chemicals were reagent-grade commercial preparations. The crystalline suspension of rabbit muscle PFK was centrifuged at 10,000 × *g* for 5 min, the pellet was suspended in 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.8, containing 100 mM KCl, 1 mM $MgCl_2$, 2 mM dithioerythritol and 1 mM EGTA (standard buffer). The enzyme solution was then dialyzed against the same buffer. PFK from bovine brain was purified by ATP-Sepharose affinity chromatography as described in [22] and stored in the presence of 50% (v/v) glycerol at −80°C. Aliquots of brain PFK were dialyzed against standard buffer before use. MAP-free tubulin from bovine brain was purified using the Weisenberg method modified by Na and Timasheff [23]. MAP-containing MTs were purified from bovine brain by the taxol-stabilizing method of Vallee [24]. The preparation contained MAPs of high molecular mass (MAP1 and MAP2) as visualized on SDS-PAGE gels.

Concentrations of muscle PFK and tubulin were determined spectrophotometrically (using the coefficients $A^{0.1\%} = 1.07$ at 280 nm [25] and $A^{0.1\%} = 1.03$ at 276 nm [23], respectively. For calculations of molar concentration of muscle PFK, the subunit molecular mass of 83 kDa was used [26]. Muscle PFK molar concentration is always given in monomers. Concentrations of brain PFK and MAP-containing or MAP-free MTs were determined by Bradford's assay [27] using the BioRad kit with bovine serum albumin as standard. Protein purity and the composition of pellet and supernatant fractions were determined by discontinous SDS-PAGE [28]. In some cases, gel electrophoresis was conducted according to [2], to obtain separation of α and β tubulin subunits.

Polymerization of MAP-free tubulin was induced in 50 mM 2-(*N*-morpholino)ethane sulfonic acid pH 6.8 buffer with 2 mM dithioerythritol. The tubulin solution (at 6–10 mg/ml concentration) was dialyzed overnight in the cold room against the above buffer, was warmed to 37°C, and taxol was added to a final concentration of 20 μM. After 30 min incubation at 37°C, MTs were pelleted for 25 min by 100,000 × *g* centrifugation at 30 °C. Microtubules were resuspended in standard buffer to a concentration of 2 mg/ml, as determined by Bradford's assay [27].

Subtilisin digestion of MAP-free MTs was performed under limiting conditions (2 mg/ml MT, 1% (w/w) subtilisin, 140 min incubation at 30°C) [29] in standard buffer but in the absence of 100 mM KCl and in the presence of 1 mM GTP. Digestion was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. Digested MTs were centrifuged (25 min at 100,000 × *g*, at 30°C), pellet was resuspended to the starting volume in standard buffer and used in binding experiments. When the effect of muscle PFK on kinetics of subtilisin digestion of MTs was studied, the above procedure was modified. 1 mg/ml MT was preincubated (30 min, 30°C) in the absence or presence of 1 mg/ml (12 μM in monomers) muscle PFK in standard buffer. Subtilisin was added to 1% (w/w) and aliquots were removed at different time points, digestion was stopped as above, and samples were analyzed by SDS-PAGE.

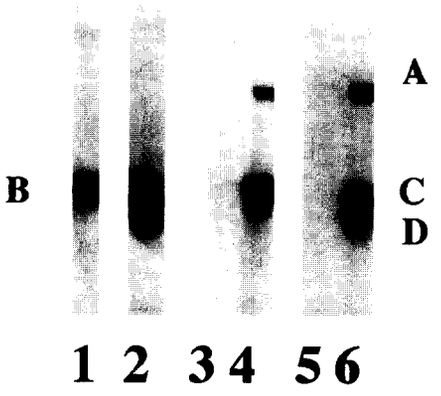

Fig. 1. Electrophoretic analysis of binding of muscle PFK to native and digested MTs. Samples were prepared as in section 2, aliquots were applied on 9% polyacrylamide gels. PFK concentration was 2.4 μM in the binding experiments. Lane 1, native MT; lane 2, digested MT; lanes 3 and 4, supernatant and pellet fractions of native MT-PFK mixtures, respectively; lanes 5 and 6, supernatant and pellet fractions of digested MT-PFK mixtures, respectively. Row A, PFK; row B, native tubulin α and β subunits; row C and D, digested α- and β-tubulin, respectively.

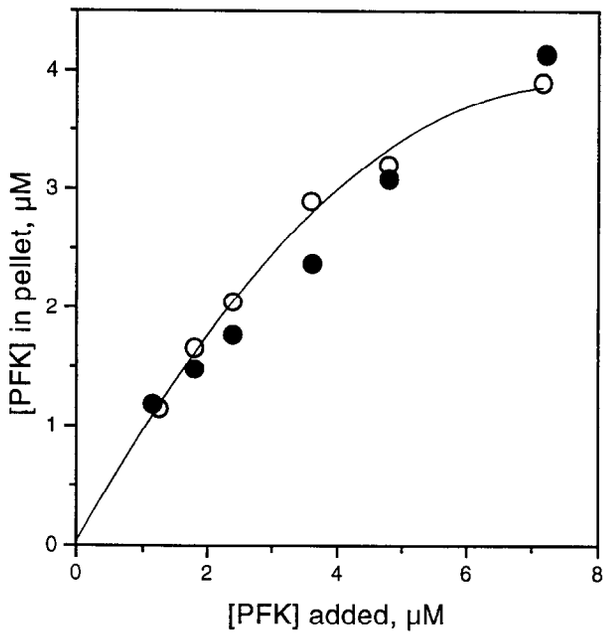

Fig. 2. Binding of muscle PFK to native (open symbols) and subtilisin treated (closed symbols) MTs. Data were obtained by densitometric analysis of pelleting experiments similar to those presented in Fig. 1. Abscissa is the PFK concentration in the incubation mixtures, ordinate gives [PFK] in the resuspended pellet, i.e. $[PFK]_{bound}$. Error of the densitometric determination was typically 10% for both data sets, as measured from three independent binding experiments at each PFK concentration.

Binding (pelleting) experiments with muscle or brain PFK and MTs (MAP-free or MAP-containing intact MTs or digested MTs) were carried out by coincubation (30 min at 30°C) of the enzyme (0.1–0.8 mg/ml) with MTs (1 mg/ml), followed by pelleting (25 min at 100,000 × *g* at 30°C). Supernatant and pellet fractions were separated, pellet was resuspended to starting volume and fractions were analyzed by densitometry of SDS-PAGE gels [13] using the BioRad GelDoc 1000 densitometer with the Molecular Analyst sofware.

For electron microscopic measurements, the pellets were fixed in 0.1 M sodium cacodylate (pH 7.4) containing 2% glutaraldehyde and 0.2% tannic acid for 1 hour at room temperature [14]. After washing with sodium cacodylate, the samples were postfixed with 0.5% OsO_4 in 0.1 M sodium cacodylate for 30 min, stained with 1% uranyl acetate, dehydrated in graded ethanol and embedded in Durcupan (Fluca, Basel). Thin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL CX 100 electron microscope at an accelerating voltage of 80 kV.

3. Results and discussion

3.1. Association of PFK with 'tail-free' MTs

Limited digestion of tubulin or MTs with subtilisin results in one cleavage at the carboxy-termini of both α- and β-subunits [2,15,29]. In our experiments we reproduced these results: digestion led to the appearance of two well separated bands at the expected molecular weight on SDS-PAGE with no significant smaller fragments (Fig. 1). In order to measure PFK binding to native and digested MTs, pelleting experiments were carried out [13]. Due to the relative large size of MT, at centrifugation

speed of 100,000 × g, MTs were pelleted with the bound enzyme, and the unbound enzyme appeared in the supernatant. Fig. 1 shows a characteristic gel pattern of a typical pelleting experiments carried out with digested and native MTs, as well. The pellets in both cases contain all MTs, visualised at molecular mass of ~50 kDa corresponding to tubulin subunits, as well as most of PFK with subunit molecular mass of 83 kDa. Fig. 2 summarizes the quantitative data obtained at six different PFK concentrations with the intact and digested MTs by densitometric analysis of the samples after SDS gel separation. There is virtually no difference between the data sets indicating that the tubulin carboxy domain is not the primary target of PFK binding.

This result is supported by another set of experiments in which the kinetics of MT digestion by subtilisin was followed in the absence and presence of PFK. The data of densitometric analysis of SDS-PAGE gels showed that PFK binding to MTs does not perturb the cleavage of the carboxy-terminal 'tails' exposed on MTs (data not shown).

Results of electron microscopic examinations of subtilisin-digested MT samples are in good agreement with the data of binding experiments. The pellets in the absence of PFK consist of randomly arranged smooth walled MTs (cf. Fig. 3a) with morphology similar to that of undigested MTs described in detail in our previous work [14]. This result is in agreement with [29]. On the other hand, addition of PFK to the subtilisin-digested MTs results in the appearance of groups of parallely aligned MTs connected by rows of periodic lateral arms (cf. Fig. 3b). Our results clearly show that if the C-terminal tubulin 'tails' were selectively removed from MTs then the overall structure of tubules is similar to the native one and modified MTs still form PFK-dependent bundles. Therefore, these results exclude the direct and dominant role of tubulin C-terminal 'tails' in the PFK binding.

Aldolase is a sequential enzyme to PFK in glycolysis that binds MT [30,31]. Knull and coworker [15] found that the removal of the tubulin carboxy-terminal peptide from MTs with limited subtilisin digestion decreased significantly the amount of aldolase copelleted with MAP-free MTs. Aldolase possesses net positive charge at physiological pH values, and complexation of aldolase and MTs is dramatically decreased by increasing the salt concentration up to the physiological one. Critical involvement of the acidic tubulin C-terminal in the aldolase–MT complex emphasizes the role of ionic interactions. Our present data suggest that other types of the interacting forces are involved in the complex formation between PFK and MTs that are virtually insensitive to the physiological ionic strength.

3.2. *MAPs are not involved in the PFK binding*

Several reports suggest the involvement of the charged tubulin C-terminal in MAPs binding to MTs [4,29,32]. On the basis of the results of the binding experiments with 'tail-free' MTs, it can be hypothesized that distinct domains of MT are the targets of PFK and MAPs. Comparative pelleting experiments were carried out with MAP-free and MAP-containing MTs and muscle PFK. A typical gel pattern (Fig. 4, inset) shows that the presence of MAPs on MTs does not interfere with PFK binding. Quantitative data at different PFK concentrations does not reveal any significant difference in the enzyme amount copelleted with MTs (Fig. 4). Thin section electron microscopic data illustrate that MTs incubated in the presence of MAPs are decorated with dense spots and filamentous projections (cf. Fig. 3c). These observations are in good agreement with earlier reports showing that MAPs are rod-like molecules and that their binding sites are helically arranged along the wall of MTs [33]. The dense spots probably represent MAPs oriented transversally to the plane of section. The MAP-decorated MTs are randomly arranged in the pellet and do not form bundles. On the other hand, many groups of closely aligned MTs connected by lateral projections appear in the samples after addition of PFK to the MAP-decorated MTs (cf. Fig. 3d). These data provide morphologic evidence that MAP-containing MTs can bind PFK in vitro and the presence of MAPs does not suppress the MT bundling activity of PFK. In vivo MAPs are integrant component of microtubular network, therefore,

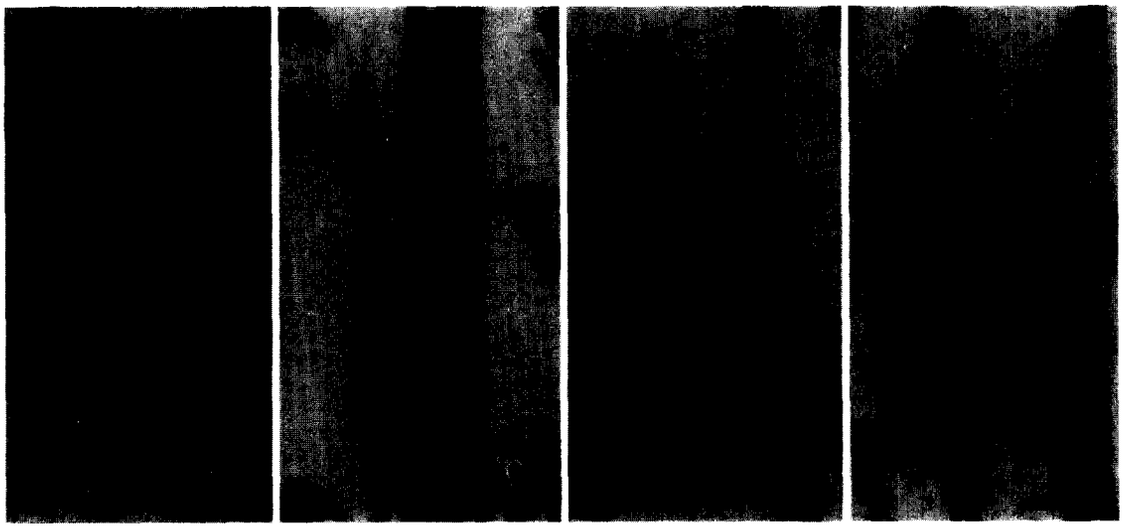

Fig. 3. Electron micrographs of subtilisin-digested (a and b) and MAP-decorated native (c and d) MTs without (a and c) and with (b and d) muscle PFK, respectively. Muscle PFK and MT concentration in the incubation mixtures during binding was 2.4 μM and 1 mg/ml, respectively. Note the presence of MT bundles connected by rows of lateral projections in the PFK containing samples. Bars: 100 nm.

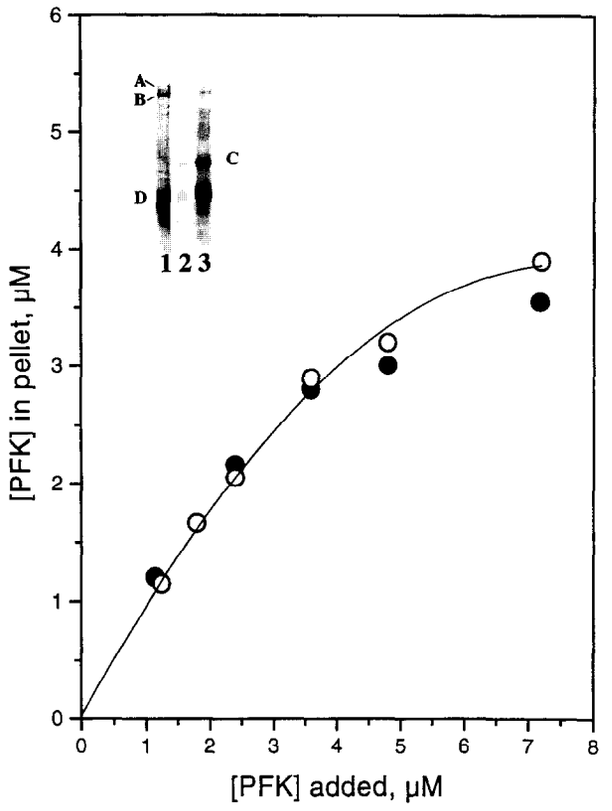

Fig. 4. Binding of muscle PFK to MAP-free (open symbols) and MAP-containing (closed symbols) MTs. Main panel: Densitometric data obtained from pelleting experiments (as in inset) are presented in the same manner as in Fig. 2. Error of determinations was 10% for the MAP-free data set, and 13% for the MAP-containing data set, as measured from three independent binding experiments at each PFK concentration. Inset: Electrophoretic analysis on 7% polyacrylamide gels of a typical MAP-MT preparation (lane 1), and supernatant (lane 2) and pellet (lane 3) fractions of a typical binding experiment at muscle PFK concentration of 2.4 μM. Rows A and B, high molecular mass MAP1 and MAP2, row C, PFK, row D, tubulin subunits.

our pioneer observation, that MAP-containing MT does bind a glycolytic enzyme is important concerning the physiological relevance of the macromolecular enzyme associations. In addition, we suggest that PFK is a unique glycolytic enzyme that specifically interacts with the MT 'body'.

3.3. *Selective association of muscle PFK to MAP-containing MTs*

The association of muscle PFK to MTs results a significant reduction of the overall activity of the kinase [13]. Concerning the physiological concentration of PFK [34], it can be expected that a significant fraction of the kinase can be complexed by MT in brain where MT concentration is especially high. On the other hand, brain and tumor cells proceed extensive glycolysis. Significant differences in the isoenzyme patterns of glycolytic enzymes exist between normal and tumor cells [20]. While individual metabolic roles have been proposed for M and L isoforms, no adequate explanation of a unique role for the C variant has yet been offered despite extensive studies into the catalytic and regulatory properties of the purified enzyme. We hypothesized that the C isoform may exhibit different affinity to MTs and thus the selective association of PFK isoforms could ensure an effective control mechanism for glycolysis.

The mixture of PFK isoenzymes was isolated from the brain extract from which MAP-containing MTs were purified. The well-established procedure of the pelleting experiments was applied for binding studies (cf. Fig. 5). While in the supernatant phase the three isoforms can be visualized, the pellet phase contains tubulin subunits with the characteristic MAP bands and an additional band corresponding to the muscle isoform of PFK (Fig. 5, inset). The ratio of isoforms in our preparation and the quantitative binding data (Fig. 5) demonstrate that the pellet (bound) phase becomes enriched in M, i.e. MTs can select among the individual isoenzymes. The muscle-type (M), but not the brain-type (C), isoenzyme extensively binds to MTs, although M and C isoforms occur in brain in comparable amount. Several differences in the properties of C and M forms could be responsible for different binding. For example, stabilities of M_4 and C_4 tetrameric structures differ significantly [35]. Since we demonstrated that the dimeric form of muscle type PFK specifically associates to MTs [13], we now suggest that differences in the nature of intersubunit forces may be primarily

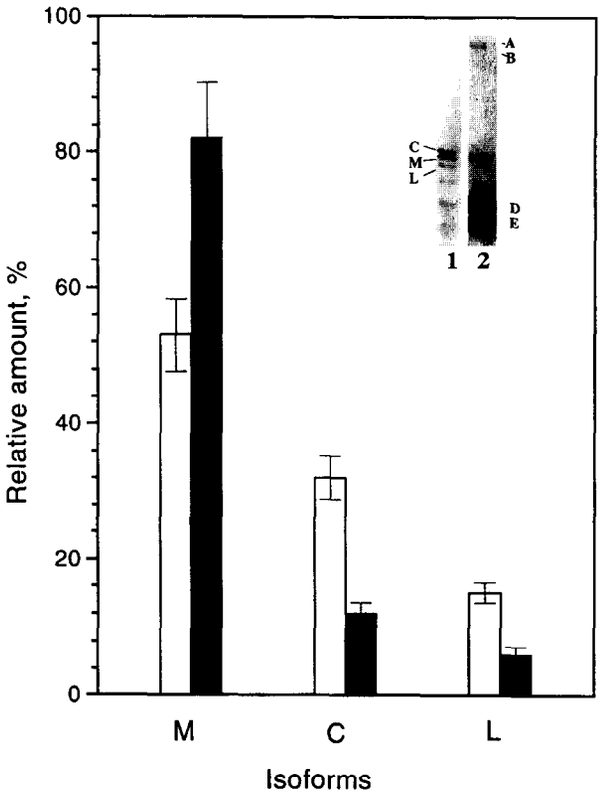

Fig. 5. Differential adsorption of PFK isoforms on MAP-containing MT. Binding conditions are given in section 2, the concentration of brain PFK preparation was 0.2 mg/ml. Main panel: Relative amount of isoforms in the brain PFK preparation (open bars) and in the pellet phase of binding experiments (closed bars) is shown (100% is the sum of the amounts of all the three isoforms). Data were obtained from by gel densitometry, mean and error of six replicates are presented. Inset: Lane 1, supernatant; lane 2, pellet phase of one typical experiment. Rows A and B, high molecular mass MAP1 and MAP2, rows C, M, L, PFK C, M and L isoforms, respectively, rows D and E, α- and β-tubulin, respectively. Electrophoresis was performed on 7% polyacrylamide gels with modifications according to [31].

responsible for the apparent selective association of PFK isoforms.

Specific enzyme associations require biorecognition of the enzyme that is based upon surface complementarity. The selective association of the inactive, dissociated form of muscle PFK to MTs but not the tetrameric form of either muscle or brain type isoenzyme provides an example for specific enzyme association that is prerequisite for its occurrence in living cell. The binding of muscle isoform of PFK to the microtubular network could provide an effective means of regulating the catalytic capacity of the enzyme, consequently controlling glycolysis. Such a mechanism might be operative in muscle and non-muscle cells. In brain and tumor cells where the isoforms of this key regulatory enzyme with different associative properties occur simultanously, the specific macromolecular associations may contribute to adjust glycolytic flux over a wide range.

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