Tubulin and microtubule are potential targets for brain hexokinase binding

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Abstract The metabolite-modulated association of a fraction of hexokinase to mitochondria in brain is well documented, however, the involvement of other non-mitochondrial components in the binding of the hexokinase is controversial. Now we present evidence that the hexokinase binds both tubulin and microtubules in brain in vitro systems. The interaction of tubulin with purified bovine brain hexokinase was characterized by displacement enzyme-linked immunosorbent assay using specific anti-brain hexokinase serum ($IC_{50} = 4.0 \pm 1.4$ μM). This value virtually was not affected by specific ligands such as ATP or glucose 6-phosphate. Microtubule-bound hexokinase obtained in reconstituted systems using microtubule and purified hexokinase or brain extract was visualized by transmission and immunoelectron microscopy on the surface of tubules. The association of purified bovine brain hexokinase with either tubulin or microtubules caused about 30% increase in the activity of the enzyme. This activation was also observed in brain, but not in muscle cell-free extract. The possible physiological relevance of the multiple heteroassociation of brain hexokinase is discussed.

Key words: Hexokinase; Microtubule; Interaction; Enzyme-linked immunosorbent assay; Immunoelectron microscopy

1. Introduction

Architecture of cytoplasm in eukaryotic cells is highly organized; it contains complex networks of the cytoskeletal filament systems, which are decorated by cytosolic proteins including glycolytic enzymes. Heterologous associations of glycolytic enzymes have been demonstrated in several cell types (for reviews see [1,2]). Indeed, subcellular particles are associated with activities of several glycolytic enzymes from brain homogenate or synaptosomes (reviewed by Knull [3,4]). Glycolytic enzymes together with several other metabolic enzymes have been identified in axonal transport in vivo as a discrete macromolecular cellular entity [5,6].

Brain hexokinase (hexokinase-I, HK), a key enzyme of the glucose metabolism, binds to the outer mitochondrial membrane [7,8] or is in the cytosolic fraction (soluble form) depending on the energy needs of the brain [9]. This interaction is sensitive to the concentrations of several ions and metabolites, specifically to the level of glucose 6-phosphate [10]. In addition, the distribution of free and bound forms of HK is species-dependent [11]. However, there are data in the literature, which suggest that in brain, HK has a greater propensity to localize at non-mitochondrial receptor sites [12,13]. In the neurons of central nervous system the majority of HK moves toward the presynaptic terminals at rates several times slower than that exhibited by the mitochondria [14], and is localized in slow component b (SCb) [15]. Data on other cell types also suggest that HK does not bind exclusively to the mitochondria. There has been prior evidence that HK can bind to microsomes, plasma membrane or cytoskeletal elements in close proximity to mitochondria [12,16–18].

In this paper the interaction of brain HK with tubulin or microtubule (MT) is detected in reconstituted system using binding and kinetic assays and is visualized by electron microscopy. The experiments have been carried out with purified brain HK and cell-free extracts prepared from brain and muscle tissues. The affinity of the binding of tubulin to HK was determined from enzyme-linked immunosorbent assay (ELISA) displacement experiments.

2. Materials and methods

2.1. Materials

Rabbit anti-HK serum and preserum were kindly provided by Prof. J.E. Wilson (Michigan State University). ATP, bovine serum albumin (BSA), NADP, NADH were purchased from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, MO, USA). Tubulin, MT and the cell-free brain and muscle extracts were prepared as described previously [19,20].

2.2. Partial purification of bovine brain HK

The purification procedure described for rat brain HK [21,22] was adapted with slight modification. Briefly, the homogenized bovine brain was centrifuged at $30\,000 \times g$ at 4°C for 30 min. Triton X-100 was added to the pellet fraction to release a significant fraction of bound HK. Triton X-100 was removed from the solubilized HK by an anion exchange chromatography on DE 52 cellulose and the bound HK was eluted by 100–150 mM KCl. Then the HK was further purified by affinity chromatography using Affi-Gel Blue column. The specific activity of the bovine brain HK preparation with $>80\%$ purity was 20 ± 2 U/mg.

2.3. HK assay

Activity of HK was assayed according to Grossbard and Schimke [23]. The experiments were carried out in HEPES buffer (50 mM, pH 7.4, containing 100 mM KCl, 10 mM $MgCl_2$, 10 mM NaH_2PO_4), 0.4 U/ml glucose 6-phosphate dehydrogenase, 1 mM NADP and 2 mM MgATP, starting the reactions with the addition of 1 mM glucose. The NADPH-production was monitored by a Cary 50 spectrophotometer at 25°C.

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Abbreviations: BSA, bovine serum albumin; HK, hexokinase-I; MT, microtubule

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2.4. ELISA experiments

Standard ELISA protocols [24] for indirect assays were used with some modifications as follows.

For standard assay, coating of plate with HK (5 μg/ml) was done for 18 h at 6°C. Incubation time was 90 min for rabbit anti-HK serum, than 90 min for the anti-rabbit antibody–horseradish peroxidase conjugate at different dilutions depending on the batches. *o*-Phenylenediamine substrate was used at 3.7 mM concentration and at 492 nm after 5 min hydrolysis time, the reaction was stopped by addition of 1 M H_2SO_4 and the absorbances were read at 492 nm on Anthos II microplate reader. Immunoreaction was not detected when preimmune serum was used instead of anti-HK serum.

For displacement experiments, tubulin at various concentrations was added to the coated HK (5 μg/ml), and preincubated with the anti-HK serum for 15 min. Subsequent steps were the same as those described for the standard assay. We used at least four parallel wells in a plate for each concentration and each experiment was repeated at least three times.

2.5. Binding to MT

Taxol-stabilized MAP (MT-associated protein)-free MT was prepared as described previously [19]. MT, purified HK or brain extract were centrifuged (40 000×*g*, 25 min, 25°C) separately, than MTs from the pellet fraction was preincubated with either purified HK or with brain extract from the supernatants in 100 mM MES buffer, pH 7.1, containing 5 mM $MgCl_2$ at 37°C for 15 min. Final concentrations of MT, brain HK, and brain extract were 2, 0.18 and 10 mg/ml, respectively. The MTs with the bound proteins were collected by centrifugation (40 000×*g*, 25 min, 37°C) and electron microscopic studies were carried out. Under these experimental conditions about 5 μg HK cosedimented with 2 mg MTs.

2.6. Electron and immunoelectron microscopy

For routine electron microscopy, MT-containing samples were fixed in a mixture of 2% glutaraldehyde, 0.2% tannic acid and 0.1 M sodium cacodylate, pH 7.4, for 1 h, postfixed in 0.5% OsO_4 and embedded in Durcupan (Fluka, Switzerland). Thin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL CX 100 electron microscope.

For immunogold electron microscopy MT preparations were fixed in 2% paraformaldehyde/0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1% sucrose and 2 mM $CaCl_2$ for 1 h, then washed, stained en bloc with 1% uranyl acetate, dehydrated and embedded in Durcupan. Sections collected on nickel grids, were etched in 5% H_2O_2 then washed in double distilled water, blocked with 0.5% non-fat dry milk powder and 1% BSA followed by incubation on a drop of rabbit anti-brain HK-IgG serum at a dilution of 1:100 in a humid chamber at 4°C overnight. After rinsing in 1% BSA in phosphate buffered saline the grids were transferred on to a drop of biotinylated goat antibody against rabbit IgG (Vector Labs., Peterborough, UK; 1:200 dilution). After washing in 1% BSA in phosphate buffered saline the grids were floated on a drop of anti-biotin gold (10 nm, Bio-Cell, Cardiff, UK; 1:100 dilution) in a humid chamber at 4°C for 5 h. Immunostained sections were examined in the electron microscope without counterstaining. Control sections were processed as described above, however, the first, HK-specific antibody was omitted.

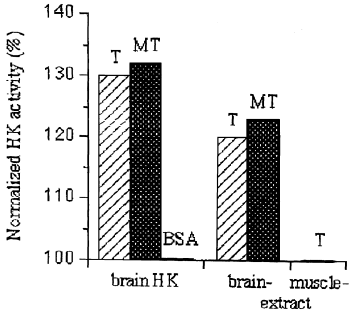

Fig. 1. Effects of tubulin, MT and BSA on the activity of HK in brain and muscle extract, and on purified brain HK. Tubulin (T) (striped bar) or MT (dotted bar) or BSA concentrations were 1 mg/ml; the concentrations of brain and muscle extracts, and brain HK were 0.8 mg/ml, 0.7 mg/ml, and 0.25 μg/ml respectively.

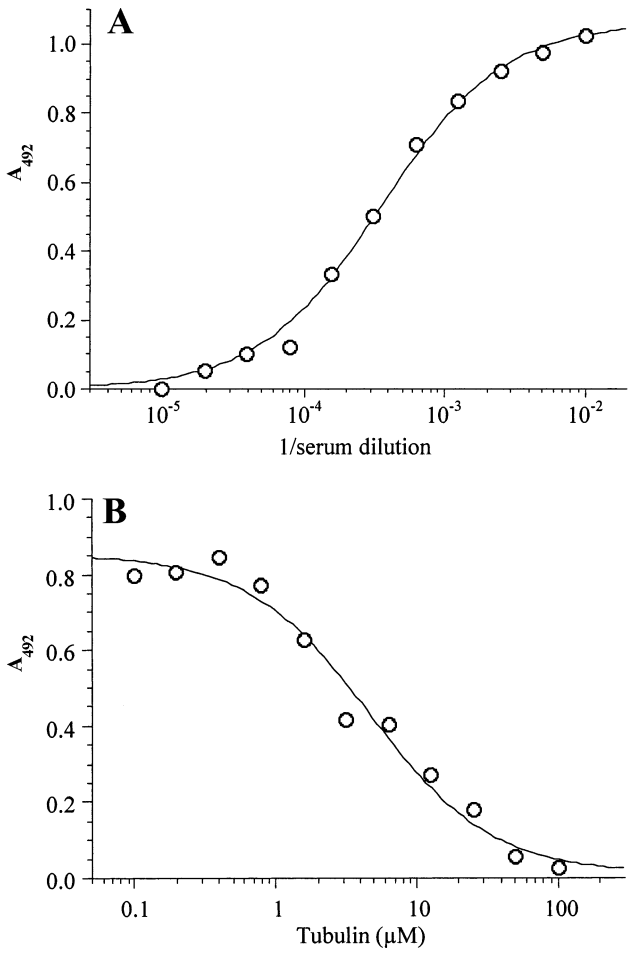

Fig. 2. A: Indirect ELISA for measuring immunocomplex formation. Absorbance was measured at 492 nm as a function of anti-HK serum dilution. B: Displacement curve of tubulin using 1000 fold diluted serum concentration.

3. Results

3.1. Activation of brain HK by tubulin or MT

In many cell types the major control step of glycolysis is the HK-catalyzed reaction. Recently, we have demonstrated that the flux of the upper part of glycolysis, from glucose to triose phosphates, controlled by HK, is increased by tubulin or MT [25,26] in the cell-free extracts of brain, but not of muscle tissue. Now we investigated the direct involvement of HK in the process.

Fig. 1 shows the effects of tubulin, MT and BSA, as an inert protein on the activity of HK in purified form and in brain and muscle extracts. In the case of purified enzyme approximately 30% activation was detected when 1 mg/ml tubulin was added to the activity assay without preincubation as compared to the control (no tubulin added). This activation was not seen by addition of 1 mg/ml BSA. The extent of activation was not further increased elevating the tubulin concentration, but it was maintained at tubulin concentration as low as 0.3 mg/ml (data not shown). As also shown in Fig. 1 MT, the assembled form of tubulin, exerts its activating

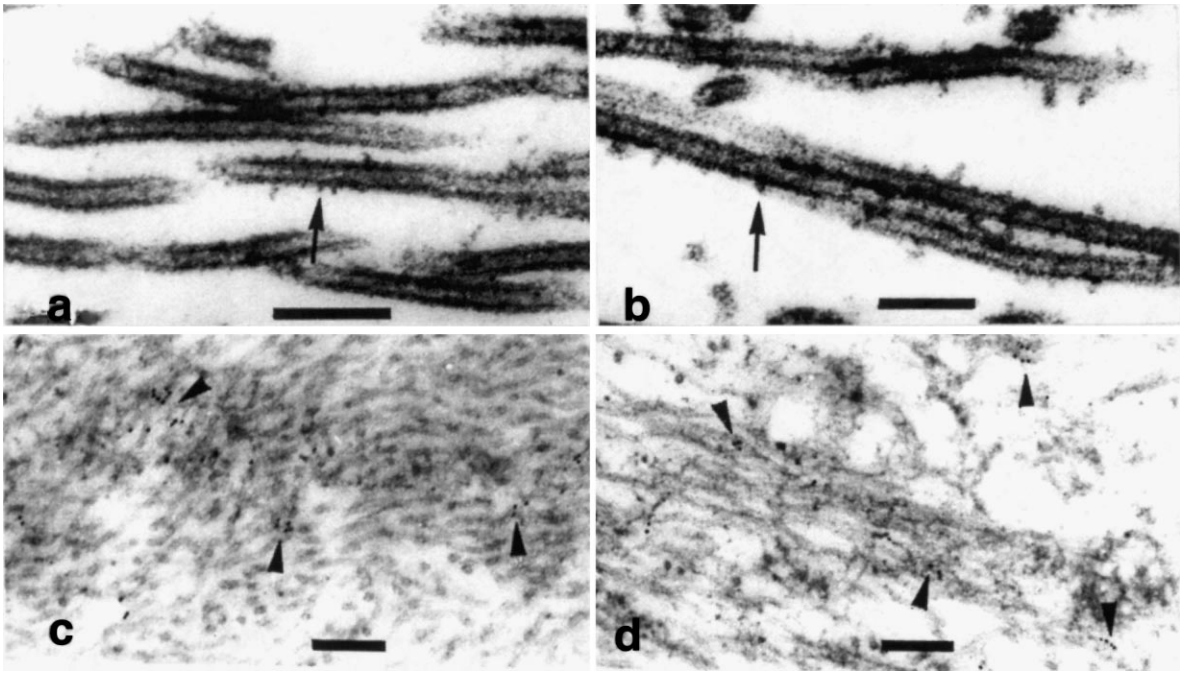

Fig. 3. Electron microscopic images of MTs incubated in the presence of purified brain HK (a, c) and brain cytosolic extract (b, d) and processed for routine electron microscopy (a, b) or immunolabelled by anti-brain HK antibody followed by biotin–gold-conjugated anti-biotin staining (c, d). Note the decoration of MTs (arrows) in (a) and (b) and the presence of 10 nm gold particles (arrowheads) on the MTs in (c) and (d). Bars: 100 nm in (a) and (b) and 200 nm in (c) and (d).

effect on brain HK as well, similarly to that obtained with tubulin.

Addition of tubulin or MTs to brain extract in which many proteins are present and known to interact with MTs resulted in an about 20% activation of HK (cf. Fig. 1). This activation was not, however, obtained when muscle extract under similar conditions was used instead of brain extract. Since the muscle contains another isoform of HK (HK-II), the negative data obtained with muscle extract or with BSA (cf. Fig. 1) indicate the relevance of the specific HK–tubulin interaction in the brain system.

3.2. Characterization of the binding of brain HK to tubulin

Displacement ELISA experiments were carried out to detect the binding of tubulin to the coated HK. Fig. 2A shows the saturation (dilution) curve measured with anti-HK serum using 5 μg/ml HK to cover the microtiter wells. The immobilized tubulin captures anti-HK antibodies from the serum, which was visualized after addition of appropriate amount of the second immunoglobulin conjugate under conditions, which ensure direct proportionality during the peroxidase reaction (A_{492}) [27]. The immunocomplex formed is characterized by $IC_{50} = 3500 \pm 700$ fold dilution.

On the basis of Fig. 2A, 1000 fold dilution of the anti-HK serum was used to detect the effect of tubulin on the immunocomplex formation with high sensitivity. The binding of antibodies to HK is inhibited by increasing the tubulin concentration as shown in Fig. 2B, and complete displacement can be reached at near 100 μM concentration. The IC_{50} value determined was 4.0 ± 1.4 μM. This value is in the same order of magnitude as we observed in the case of other glycolytic enzymes [28,29].

The effects of glucose 6-phosphate and MgATP on the HK–tubulin interaction were also tested by ELISA, under similar conditions as described for Fig. 2B. We found that the presence of these metabolites at 1 mM concentrations did not cause significant perturbation of HK–tubulin interaction (data not shown). The finding that the specific metabolites of HK did not induce the dissociation of the HK from tubulin suggests that other interactive forces are involved in this heteroassociation than those observed in the case of HK binding to mitochondria [10].

3.3. Visualization of HK on MT

To study the morphologic background lying behind the binding of HK to MT and the enzyme activation, HK-bound MT samples were prepared and visualized by electron microscope. The MTs were pelleted in the presence of purified HK or brain extract with appropriate sedimentation velocity to separate the free and the bound proteins [28]. The pellets prepared without addition of HK or brain extract contain more or less parallel-aligned smooth-surfaced MTs of about 25 nm of diameter as we demonstrated previously [30]. However, a very fine, sparse decoration is seen on several MTs made with addition of HK (Fig. 3a). When MTs cosedimented with brain cell-free extract were visualized heavy deposits of electron dense aggregates appeared on the MTs (Fig. 3b) due to the binding of several cytosolic proteins as we have reported previously [26,31]. To see whether the decorating material contains HK, immunogold labelling was applied to samples of MTs incubated either in the presence of purified brain HK (Fig. 3c) or brain extract (Fig. 3d). As shown in these figures, immunogold conjugates are clearly present on the MTs. No immunostaining is seen on MTs made without addition of HK or brain extract, and non-specific labelling is negligible on control sections incubated in the absence of first

antibody (data not shown). Therefore, the electron microscopic images provide evidence that MTs and HK recognize each other, even in high excess of other cytosolic proteins.

4. Discussion

Our present observations that the brain isoform of HK can bind to tubulin and MT and the binding causes the activation of HK in vitro in brain systems suggest that the microtubular network could be a potential target for HK binding. The negative results (no interaction) of control experiments carried out with muscle extract, which contains another isoform of HK or with BSA suggest the specific character of the heteroassociation of brain HK. This heteroassociation could be important from structural and physiological point of views, since in neuronal cells the concentration of MT, the major component of the axons, is very high, and HK plays a key role in the control of the glycolysis; especially in brain where glucose is the major energy source. It is well documented that a fraction of HK, reversibly associated with the mitochondrial membrane, is releasable specifically by glucose 6-phosphate, which results in the conversion of the active HK species into a less active form [32]. When associated with mitochondria, the affinity of the enzyme for ATP is elevated and sensitivity to inhibition by glucose 6-phosphate is lowered [10]. There are data, on the other hand, which show the significant sequestration of HK by non-mitochondrial cell components including membranes of endoplasmic reticulum/microsomes [18], actin filaments [18] and other cytoskeletal components [14]. Our present data support this idea and provide evidence for the binding of brain HK to both tubulin and MTs. The functional consequences of these associations are probably far-reaching. The kinase is activated due to this heteroassociation as in the case of mitochondrial binding, however, this interaction is glucose 6-phosphate and MgATP-independent. The fact, that HK is allosterically regulated by a couple of specific ions and metabolites, and a fraction of the enzyme occurs in particle-bound forms which exhibit different catalytic properties, indicates that the energy state of brain cell is finely controlled by multiple equilibrium in which the microtubular system may be involved. Increased fluxes through HK/glucose 6-phosphate dehydrogenase coupled enzymatic reactions in the presence of brain tubulin and MTs were reported by Cortassa et al. [33]. Several reports suggest that the MTs may interact with mitochondria via binding MT-associated proteins (MAP2) to the pore-containing domain of the outer mitochondrial membrane [34,35]. These observations strengthen the view that fine interplay may exist between these two cell components in the regulation of location and activity of HK. The association of HK to MTs may regulate the susceptibility of the enzyme to degradation. This suggestion is based on the observation of Magnani et al. [13] who showed that the soluble but not mitochondria-bound HK is a good substrate of the ubiquitin-dependent proteolytic system.

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