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Microtubular Protein in Its Polymerized or Nonpolymerized States Differentially Modulates In Vitro and Intracellular Fluxes Catalyzed by Enzymes of Carbon Metabolism

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Abstract The fluxes through HK/G6PDH and PK/LDH coupled-enzymatic reactions were quantified in the presence of physiological concentrations (1–15 μM) of polymerized or non-polymerized microtubular protein (MTP) from rat brain and in a permeabilized yeast cell system. In vitro enzymatic fluxes were increased by either polymerized or nonpolymerized brain MTP mainly in the lower range of MTP concentration. At fixed MTP concentrations in the flux stimulatory range of HK/G6PDH (1 mg/ml MTP) or PK/LDH (0.4 mg/ml MTP), a hyperbolic and sigmoidal response to NADP and PEP, respectively, was detected. That dependence varied according to the polymeric status of MTP. The specificity of the phenomenon observed in vitro, was tested for the PK/LDH and HK/G6PDH enzymatic couples in the presence of neutral polymers such as glycogen ($\leq$10 mg/ml), poly(ethylene glycol) (up to 10% w/w) or G-actin ($\leq$1 mg/ml). In permeabilized *Saccharomyces cerevisiae* cells, the PK-catalyzed flux was sensitive to microtubule disruption by nocodazole (15 μg/ml). The HK/G6PDH system was not affected by nocodazole showing values of kinetic parameters close to those obtained in vitro in the presence of polymerized brain MTP. Indirect immunofluorescence with specific antibodies against tubulin allowed to confirm the microtubules disruption in the presence of nocodazole in permeabilized yeast cells under the same conditions in which enzymes were assayed intracellularly. The experimental evidence is in agreement with the observed phenomenon of increase in fluxes in the enzymatic reactions assayed to be specifically induced by MTP either in vitro or in situ. The results presented are discussed in terms of the assembly of large supramolecular structures as a supraregulatory mechanism of synchronization of systemic cellular processes such as metabolic fluxes.

Key words: microtubular protein, microtubules, carbon metabolism, flux regulation, permeabilized yeast cells

INTRODUCTION

A challenging question in biology and biochemistry is how a change in environmental parameters such as the presence of light, hormones, pH, and oxygen could induce a generalized cellular response that leads the cell to different developmental paths, to stop division or to redirect metabolic fluxes. For many intracellular signal transducing processes, molecular second messengers that affect different cellular activities and their mechanisms, have been characterized [Berridge, 1987].

The transition from the primary molecular mechanism elicited by an environmental signal to the generalized cellular response at a higher level of organization may be visualized as an instability in the dynamics of the processes involved at different levels of organization [Aon and Cortassa, 1993]. The task is then to identify processes whose dynamics being triggered by

Abbreviations used: MTP, microtubular protein; MES, 2-(N-morpholino)ethanesulfonic acid; MAPs, microtubule-associated proteins; EGTA, ethyleneglycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid; PIPES, Piperazine-N,N'-bis[2-ethane-sulfonic acid]; 1,4-piperazine-diethanesulfonic acid; HK, hexokinase; G6PDH, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; FDP, fructose 1,6-bisphosphate; PEG, poly(ethylene)glycol; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

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environmental signals could be transduced in a coherent cellular response emerging at higher levels of organization. We previously suggested [Aon and Cortassa, 1993] that the appearance of macroscopic coherence at the cellular level revealed as new functional properties, would occur in processes at spatial dimensions of micrometers and relaxing in the order of minutes, e.g., microtubules may reach lengths in the order of micrometers and depolymerize within minutes [Kirschner and Mitchison, 1986; Erickson and O'Brien, 1992]. We hypothesize then that polymerization-depolymerization of cytoskeleton components may be involved in the emergence of coherence between different cellular activities.

The interests of several workers before has been raised by the elucidation of enzyme binding properties with actin, its effect on enzyme kinetic properties, and the interaction between enzymes in supramolecular aggregates and its influence upon transition times in consecutive catalytic steps [Clarke et al. 1985; Masters, 1984; Luther and Lee, 1986]. Changes in the kinetic properties of glycolytic enzymes have been reported upon binding to actin filaments [Masters, 1984; Luther and Lee, 1986; Keleti et al. 1989]. However, the assembly-disassembly of cytoskeleton components as a macroscopic, coherent, inducer of changes in systemic properties such as cellular fluxes has not yet been addressed.

In the present work we have mainly focused upon the in vitro and intracellular global effects of the presence of polymerized or nonpolymerized microtubular protein on fluxes through two chained reactions catalyzed by enzymes of carbon metabolism. We approached the supraregulatory effects of microtubular protein assembly-disassembly by first, establishing if, and to what extent, are regulated the fluxes through those coupled enzymatic reactions both, in vitro and in a permeabilized yeast cell system and, secondly, by investigating the effect upon kinetic parameters. The results obtained show that on the one hand, the presence of assembled or unassembled microtubular protein in the physiological concentration range appears to increase the flux through both enzymatic reactions tested although at a different concentration range; and on the other hand, that enzymatic reactions able to interact or not with the microtubular lattice showed either sensitivity or not, respectively, to the state of intracellular organization of microtubules.

METHODS

Microtubule Protein Preparation

The preparation of MTP from rat brain by two assembly-disassembly cycles was performed according to Arce and Barra [1983]. The microtubular protein was stored as pellets at −70°C before assay. Brain MTP was further purified in order to separate MAPs from tubulin through a phosphocellulose column by the method of Weingarten et al. [1975]. Fractions eluting in the void volume, identified as tubulin, or those eluted with 0.8 M KCl were pooled, desalinated and concentrated by lyophilization. The lyophilized samples were resuspended in buffer 0.1 M MES, 1 mM EGTA, pH 6.9 before assay.

Polymerization of Brain Microtubular Protein

MTP polymerization was monitored by absorbance at 350 nm in a cuvette thermostated at 37°C. Polymerization was performed in a final volume of 0.5 ml containing 0.1 M MES, 1 mM EGTA, pH 6.9, 2 mM $MgCl_2$, 2 mM GTP and MTP at the concentrations indicated in each case before the enzymatic assay was carried out. Similar polymerization kinetics of MTP was observed when performed in the absence or in the presence of a GTP-regenerating system with a final concentration of 10 mM Na-acetyl phosphate plus 1 U ml^{-1} of acetate kinase. MTP or tubulin polymerization performed in the presence of PIPES-based buffer gave similar results to those obtained in MES-based buffer.

Enzyme Activity Determinations In Vitro

All enzyme activities were monitored through the change in absorbance at 340 nm in a cuvette thermostated at 37°C. When indicated brain MTP was added to the incubation system at final concentrations between 50 to 2,000 μg/ml. GTP was added at 2 mM when indicated. When enzymatic fluxes were determined in the presence of polymerized MTP, we first recorded at 350 nm the kinetics of assembly of MTP and after the pseudo-steady state was reached the enzymatic mixture was added and the activity monitored at 340 nm as a function of time. No differences in activity were observed when either polymerization was made in the presence of enzymes or added after polymerization. The amount of enzyme protein used corresponded to the linear range of activity. In the interaction experiments of both enzymatic couples (see text and legend to Fig. 3), the MTP pellets obtained

after centrifugation at 40,000*g*, 37°C, for 2 h, were resuspended by mild pipetting on ice.

The incubation systems of each enzymatic assay were as follows:

HK/G6PDH: The procedure used was based on a modification of the assay described by Aon et al. (1989). The incubation mixture contained in a final volume of 0.5 ml: 0.1 M MES, pH 6.9; 1 mM EGTA, 2 mM $MgCl_2$, 1 mM glucose, 1 mM NADP, 1.5 mM ATP, 0.75 mU hexokinase and 0.37 mU glucose 6 phosphate dehydrogenase.

PK/LDH: This was assayed with a modification of the procedure described by Czok and Eckert (1965). The incubation mixture contained in a final volume of 0.5 ml: 0.1 M MES, pH 6.9; 1 mM EGTA, 2 mM $MgSO_4$, 100 mM KCl, 10 mM phosphoenol pyruvate, 1 mM NADH, 1 mM ADP, 42 mU pyruvate kinase and 1.42 U lactate dehydrogenase.

Additional Controls

In preliminary experiments we tested that our brain MTP preparations were devoid of contaminating activities related to the enzymatic couples assayed. We did the latter by adding the enzymes' substrates to MTP samples in the absence or in the presence of GTP. No activity was detected either for HK/G6PDH or PK/LDH as well as for PFK/PK/LDH or glutamic dehydrogenase paths (not shown).

Enzymatic Fluxes Determination in the Presence of Neutral Polymers and Actin

G-actin (0.25–1.0 mg/ml or 5.95–23.8 μM as monomer) was polymerized as in Suzuki et al. [1989] in the presence of the incubation mixture of HK/G6PDH and PK/LDH, except for glucose (former couple) or NADH/PEP (latter couple) which were added after completion of the polymerization step. Actin polymerization was monitored at 350 nm and a pseudo steady state was achieved after ~30 min. Enzyme activity was monitored as described above.

After completion of the enzymatic reaction, the whole content of the cuvette was centrifuged for 2 h at 40,000*g* and 30°C in order to investigate if the enzymes were interacting with the actin bundles. The actin pellets were resuspended by mild pipetting and the same incubation mixture with exception of the enzymes was added. The activity was monitored as described above. Both enzymatic couples were assayed as described above in the presence of glycogen (1–10 mg/ml) or PEG 8000 (1–10% w/w).

Permeabilization of *Saccharomyces cerevisiae* Cells

The *Saccharomyces cerevisiae* haploid strain A364A (MATa, ade1, ade2, ura1, his7, lys2, tyr1, gal1) was obtained from the Yeast Genetic Stock Center (MCB/Division of Biophysics and Cellular Physiology, University of California, Berkeley). Yeast cells were grown in YNB (yeast nitrogen base) medium plus 1% glucose supplemented with adenine (25 μg/ml), uracil (20 μg/ml), his (20 μg/ml), lys (34 μg/ml), tyr (25 μg/ml), arg (20 μg/ml), thr (20 μg/ml), trp (25 μg/ml) and leu (15 μg/ml) as described before [Aon et al. 1991].

Yeast cells were harvested from exponentially growing batch cultures under aerobic conditions at 25°C, resuspended at an OD_{540} of 1.0 (8.5×10^6 cells per ml) and permeabilized as described by Serrano et al [1973]. After permeabilization, the cell suspension was centrifuged and the pellet resuspended in buffer 0.1 M MES, pH 6.9 containing 1 mM EGTA and stored on ice before use.

Intracellular Enzyme Activity Measurements

In situ HK/G6PDH and PK/LDH fluxes and kinetic behavior were measured as described for in vitro determinations but with slight differences. Twelve μl of the permeabilized yeast cell suspension described above was used as the enzymatic source and, only the substrates added in each of the enzymatic couples. Antimycin A (4 μM) was added to the incubation mixture for the assay of PK since in the absence of PEP there was a substantial reoxidation of NADH that was independent of PK's activity. The addition of the respiratory inhibitor antimycin A annihilated 90% of the NADH reoxidation activity independent of PK without affecting the latter, suggesting then that the former was dependent on mitochondrial electronic transport. The residual NADH reoxidation (10%) was discounted by running control samples in the absence of PEP.

The amount of cell suspension used was tested in several cell batches to be in the linear range of the enzymatic activity under assay. In preliminary experiments we first checked that intracellular enzymes were directly accessible to external substrates and remained within the boundaries of the cell wall in toluene/ethanol permeabilized *S. cerevisiae* strain A364A.

For the intracellular assay of enzymatic fluxes in the presence of nocodazole (15 μg/ml) dis-

solved in ethanol or DMSO (Jacobs et al., 1988), or taxol (1 μM dissolved in DMSO), yeast cells harvested from exponentially growing batch cultures and washed were preincubated in the presence of both drugs. After 30-min exposure, the yeast cell suspension was permeabilized. Yeast cells were centrifuged and resuspended in buffer 0.1 M MES, pH 6.9 containing 1 mM EGTA and 15 μg/ml of nocodazole or 1 μM taxol and stored on ice before use. Intracellular flux determinations in permeabilized control cells were carried out in the presence of identical amounts of DMSO as those utilized to dissolve nocodazole or taxol.

Protein Determination

Protein was determined according to the method of Lowry using bovine serum albumin as standard.

Fluorescence Staining of Microtubules and Fluorescence Microscopy of Permeabilized Yeast Cells

After permeabilization, yeast cells were fixed and its cell wall digested as described by Pringle et al. [1991]. Microtubules were visualized by indirect double immunofluorescence with specific antibodies (clone DM1A, Sigma Chemical Company, St. Louis, MO) according to the procedure described by Cáceres et al. [1992], except for the step in which the fixed cells were treated with methanol and acetone instead of Triton X-100. Fluorescence staining of microtubules in permeabilized control cells was carried out in the presence of identical amounts of DMSO as those used to dissolve nocodazole or taxol. The integrity of yeast spheroplasts after permeabilization and fluorescence staining was assessed by DIC microscopy.

Materials

Methyl-(5[2-thienylcarbonyl]-1H-benzimidazol-2-YL)carbamate (Nocodazole), G-actin (from bovine muscle), PEG 8000 and polylysine were purchased from Sigma Chemical Co. (St. Louis, MO). Taxol was from Calbiochem.

RESULTS

Effect of Brain MTP Concentration in Its Polymerized or Nonpolymerized State on HK/G6PDH and PK/LDH Fluxes and In Vitro Kinetic Properties

To address the question if MTP might exert regulatory effects on enzyme-catalyzed fluxes, we assayed the catalytic activity of enzymes from representative reactions of carbon catabolism at different MTP concentrations either in its polymerized or nonpolymerized state. Under the polymerization conditions used in the present work, 75% of the protein assembles for MTP concentrations higher than 1 mg/ml as could be judged from the amount of protein spun down after 2 h at 40,000*g* at 37°C. Forty percent and 70% of the protein polymerized at 0.4 and 0.8 mg/ml of MTP, respectively. At first, we standardized the assays for two coupled enzymatic systems, such as HK/G6PDH and PK/LDH, under conditions in which tubulin can polymerize, i.e., in buffer MES-EGTA, $MgCl_2$, pH 6.9 or PIPES-buffer (see Methods). For the enzyme couple HK/G6PDH, the kinase was in excess with respect to G6PDH and saturated for its substrates, glucose, NADP and ATP. The flux through the enzymatic couple was then proportional to the activity of G6PDH. The latter was proved by showing the insensitivity of the flux to ATP (not shown). In the system PK/LDH, the dehydrogenase was in excess with respect to the kinase. The flux observed was reflecting the activity of PK and its allosteric properties were put in evidence (see last paragraph of this section).

In all cases, controls were run in parallel in the presence (to compare with the effect of microtubules) or in the absence (to compare the effect of nonpolymerized MTP) of GTP. Nonpolymerized MTP increased to a maximum of 2.4-fold (or 140%) the flux through the HK/G6PDH enzymatic system relative to the control in the absence of MTP (Fig. 1A). The flux through the enzymatic couple PK/LDH exhibited a maximum 2.5 fold (or 150%) increase in the range of MTP concentration of 0.2 to 0.4 mg/ml (Fig. 1B); the activity decreasing upon a further increase in MTP concentration to an average level of 1.7 times (or 70%) the activity of the control. In the presence of polymerized MTP, the flux through HK/G6PDH increased by 1.6-fold relative to the control, showing flux-saturation for MTP concentrations higher than 0.8 mg/ml (Fig. 1A). On the other hand, the presence of microtubules provoked a linear increase in flux of the PK/LDH coupled reaction, up to values of 2.8 at 1.5 mg/ml MTP (Fig. 1B).

The results obtained with HK/G6PDH in the presence of either nonpolymerized or polymerized MTP were not modified by the presence of an asters' preparation from *Spisula* sp espermatozoa (not shown). We then fixed the MTP con-

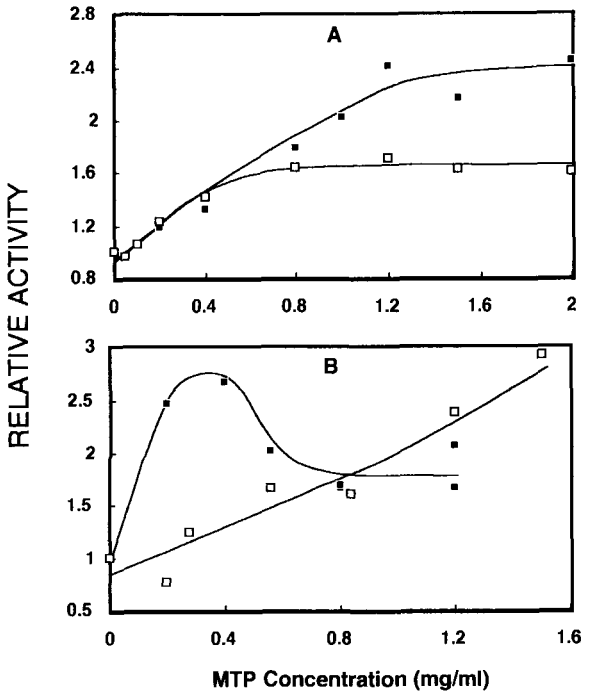

Fig. 1. Effect of the presence of brain microtubular protein in its polymerized or nonpolymerized state on enzymatic fluxes. In (A), the activity of the enzyme couple HK/G6PDH is plotted as a function of MTP concentration respect to the activity of the couple in the absence of MTP. The activity measurements in the presence of polymerized MTP were referred to a control assayed in the presence of 2 mM GTP. The increase in relative activity was referred to the control: activity 1.0. (B) corresponds to the activity of the PK/LDH system. Filled and open squares are used for the activity in the presence of nonpolymerized and polymerized MTP, respectively. The conditions of activity assay are those described under Methods. Each point represents the mean of duplicates from two independent experiments. The lines joining the experimental points were drawn by eye.

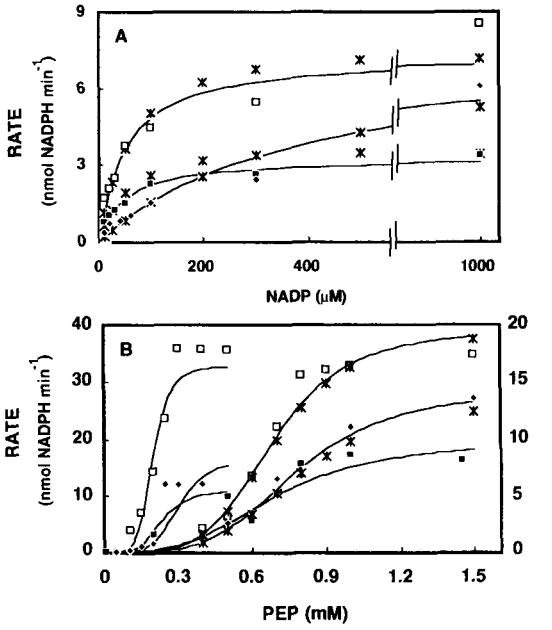

Fig. 2. Kinetic parameters of HK/G6PDH and PK/LDH in the presence of polymerized or nonpolymerized brain MTP. In the y-axis the initial rates of enzymatic fluxes in the absence of MTP (■) or presence of polymerized (◆) or nonpolymerized MTP (□) in the absence (B: right curves, right axes) or presence (B: left curves, left axis) of 10 μM FDP, are plotted as a function of NADP (A) and PEP (B) substrates for the HK/G6PDH (1 mg/ml of MTP) and PK/LDH (0.4 mg/ml of MTP) systems, respectively. The continuous lines represent the fitting to the experimental data using the equations with their corresponding kinetic parameters shown in Table I.

centration in the flux-stimulatory range and varied the substrate concentration of the limiting enzyme, i.e., NADP or PEP for HK/G6PDH or PK/LDH couples, respectively. The presence of 1 mg/ml of polymerized or nonpolymerized brain MTP provoked changes in kinetic parameters of the assayed G6PDH coupled to hexokinase (Fig. 2A). An 8-fold increase in the K_M of G6PDH for NADP and a two-fold increase in V_{max} with respect to the control without MTP, was induced in the presence of polymerized MTP; nonpolymerized MTP only induced a two-fold increase in V_{max} (Table I).

The results obtained with the coupled PK/LDH system as a function of PEP showed that the presence of polymerized brain MTP induced an increase in cooperativity as measured by n (Hill coefficient), from n = 3.31 ± 0.35 to n = 4.08 ± 0.15 and an increase in V_{max} of 44% with respect to the control (Fig. 2B; Table I). Furthermore, nonpolymerized MTP induced an even higher increase in cooperativity (from n = 3.31 ± 0.35 to n = 4.48 ± 0.31) and a 100% increase in V_{max} (Fig. 2B; Table I). The change in kinetic parameters of PK could be mechanistically induced by interaction of the enzyme(s) with MAPs (see next section).

Interaction Between Enzymes and the Microtubular Lattice In Vitro

To establish whether the enzymes do interact with the microtubular structure we designed the following experiment: brain MTP (1 mg/ml) was polymerized in the presence or absence of either the enzyme couple HK/G6PDH or PK/LDH (see legend Fig. 3). Polymerization was followed by incubation at 37°C for 30 min in the presence of the enzymes. After incubation, the system was centrifuged at 37°C and 40,000*g* for 2 h and the pellet and supernatant were investigated for the catalytic activity of the enzymatic couples. The main constituent of the pellet was tubulin as could be judged by SDS-PAGE (not shown). The activity of the couple HK/G6PDH was entirely recovered in the supernatant after

TABLE I. Effect of Polymerized or Nonpolymerized Brain MTP on HK/G6PDH and PK/LDH Kinetic Parameters*

	−MTP	+MTP	+Polym. MTP
HK/G6PDH			
V_{max} (nmol/min)	3.27 ± 0.21	7.32 ± 0.82	7.68 ± 1.75
K_M (μM)	46 ± 7.5	50.6 ± 1.36	392 ± 150
PK/LDH			
V_{max} (nmol/min)	9.79 ± 0.64	19.52 ± 0.94	14.14 ± 0.52
$K_{0.5}$ (mM)	0.685 ± 0.101	0.682 ± 0.047	0.773 ± 0.049
n	3.31 ± 0.35	4.48 ± 0.31	4.08 ± 0.15

*The data presented in Figure 2 were fitted with an equation describing Michaelis-Menten kinetics (eq. 1) for the activity of HK/G6PDH (Fig. 2A) and with a generalized Hill equation (eq. 2) for the PK/LDH activity (Fig. 2B). The values of the kinetic parameters are ±SD. The fitting procedure was performed with Enzifitter by R. J. Leatherbarrow (Elsevier, Biosoft) and a Levenberg-Marquardt algorithm. Both fitting procedures comprise non-regression analysis of the data according to the following equations:

$$V = \frac{V_{max}[NADP]}{K_m + [NADP]} \quad \text{(eqn. 1)}$$

$$V = \frac{V_{max}[PEP]^n}{(K_{0.5})^n + [PEP]^n} \quad \text{(eqn. 2)}$$

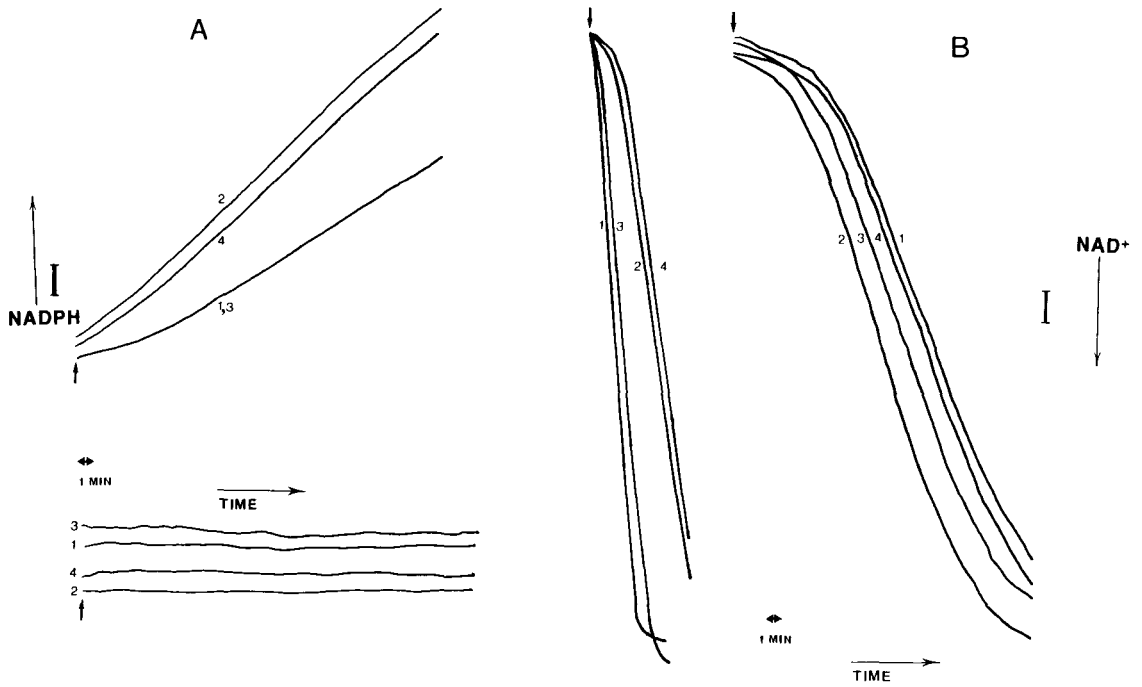

Fig. 3. Interaction of HK/G6PDH and PK/LDH with brain MTP. **A** and **B** show the kinetics of change in NAD(P)H absorbance in the supernatant and pellet after centrifugation of polymerized MTP (1 mg/ml) incubated with the enzymatic couples HK/G6PDH (A) or PK/LDH (B). Upper and lower traces in A, and left and right traces in B correspond to the activities of the supernatant and pellet, respectively. Numbers 2 and 4 indicate the presence of ATP and NADP in the incubation medium for HK/G6PDH (A), and the presence of ADP for PK/LDH (B). Numbers 1 and 2 indicate that the polymerization of the MTP was performed in the presence of the enzymes while in 3 and 4 the incubation with the enzymes was performed after the polymerization step. The absorbance scales are 20 μM NADPH (A) and 17 μM NADH (B). Arrows point to the start of the enzymatic assay.

centrifugation regardless the polymerization proceeding in the presence of enzyme protein (Fig. 3A). Irrespective of the presence of enzyme during the polymerization step or only in the following 30 min incubation, the activity of the couple PK/LDH was distributed among pellet (19%) and supernatant (81%) (Fig. 3B). Taking into account that the volume of the pellet was one fiftieth (1/50) of the total volume and that one fifth (⅕) of the activity remained bound to the

pellet, the microtubular pellet is ten times more concentrated in enzyme protein with respect to the supernatant concentration. This result indicates binding of the enzymatic protein to the microtubular structure that exerts regulatory actions on activity as shown above (Figs. 1B, 2B). We further investigated whether the increase in fluxes in the PK/LDH and HK/G6PDH systems was mediated by either MAPs or tubulin or both. After purification of brain MTP we tested, MAPs and tubulin both separately or reconstituted. The results are summarized in Table II and they clearly show that the activation of fluxes sustained by the PK/LDH system is MAPs-mediated. Purified brain tubulin polymerized in the presence of 10 μM taxol or in its absence either slightly inhibited or did not affect the relative activity of the PK/LDH enzymatic couple. When isolated brain MAPs were added to the incubation system, the two fold activation by nonpolymerized brain MTP was recovered (Table II). Furthermore, the reconstituted system (MAPs plus tubulin) reproduced the extent of flux increase of brain MTP without further purification either in its polymerized or nonpolymerized states (Table II). In the case of the HK/G6PDH system, it appears that the activation effect on fluxes was due to the ensemble of brain MTP and not to its isolated components (Table II).

Effect of Actin and Neutral Polymers Concentration and Polymerized-Actin on HK/G6PDH and PK/LDH Fluxes

To investigate whether the effect on enzymatic fluxes was elicited by any polymer or any negatively charged polymer we tested the effect of actin, neutral polymers (glycogen) or the inert synthetic PEG 8000 on enzymatic fluxes. The PK/LDH and HK/G6PDH reactions were analyzed in the presence of either up to 10% PEG (w/w) or 10 mg/ml of glycogen. None of the polymers assayed affected the flux through the HK/G6PDH couple (results not shown). Glycogen was slightly inhibitory (20%) of PK/LDH flux at concentrations higher than 4 mg/ml. The inhibitory effect did not increase up to 10 mg/ml glycogen. PEG, at concentrations ranging from 4% to 10%, did not induce changes in the enzymatic fluxes sustained by PK/LDH (results not shown). These results are consistent with the effects shown by brain MTP on enzymatic fluxes to be unlikely due to locally high concentrations of polymers.

The molecular crowding effect promoted by glycogen and PEG described above inhibited rather than increased enzymatic fluxes. We further investigated if actin (as tubulin, a negatively charged polymer; isoelectric point ~5.5), polymerized under conditions of molecular crowding (mimicked by 5% PEG w/w), could promote similar effects as those shown by polymerized-MTP. Actin filaments polymerized after 30 min at concentrations higher than 0.5 mg/ml in the presence of PEG ≥5% (w/w) pack themselves as loose bundles which constitutes a structure readily to be formed in the cytoplasmic matrix [Suzuki et al., 1989]. The effect of actin concentrations from 0.25 to 1.0 mg/ml (5.95 to 23.8 μM of actin monomer) was tested on both enzymatic couples. Actin slightly inhibited (10%) the flux through the HK/G6PDH couple, and significatively (~40%) the flux sustained by the PK/LDH system (results not

TABLE II. Effect of Tubulin and MAPs Isolated Components From Brain MTP on PK/LDH and HK/G6PDH Fluxes*

Protein assayed	Concentration (mg/ml)	Polymerization	Relative Activity PK-LDH	Relative Activity HK-G6PDH
MTP	1	Yes	2	1.7
MTP	1	No	1.7	2
Tubulin (+10 μM taxol)	1	Yes	0.76	1.17
Tubulin	1	No	1.05	1.41
	0.6	No	1.06	—[a]
Tubulin	1	Yes	0.84	—
MAPs	0.11	No	2.04	1.035
Tubulin + MAPs	0.5 + 0.05	Yes	1.74	—
Tubulin + MAPs	0.5 + 0.05	No	2	—

*Enzymatic fluxes were measured in the presence of polymerized or nonpolymerized MTP, purified tubulin, MAPs, or MAPs plus tubulin at the concentrations indicated in the 2nd column as described in Methods. Under all conditions and previous to the enzymatic determinations, tubulin or MTP assembly was monitored as described under Methods.
[a]Not determined.

shown). Since controls were run in parallel in the presence of the same concentration of PEG but in the absence of actin, the inhibition of the PK/LDH system was due to the presence of actin. Furthermore, when the PEG concentration used was higher than 4% up to 10% w/w, we were not able to detect changes in the PK/LDH flux (see previous paragraph). Under the same experimental conditions we investigated if both enzymatic systems were interacting with the actin bundles. After incubation, the enzymatic reactions that proceeded at different actin concentrations were centrifuged and the enzymatic activity remaining in the pellet measured as described under Methods. The activity of the enzymatic couples under study were entirely recovered in the supernatant at all the actin concentrations tested (not shown). The results presented in this section are in agreement with the effect of flux-regulation induced by (polymerized-) brain MTP being specific of MTP.

Cellular Correlate of MTP-Elicited Modulation of PK/LDH and HK/G6PDH Enzymatic Fluxes

The yeast *Saccharomyces* is a single-celled eukaryote which contains tubulin and actin in its cytoskeleton [Solomon, 1991]. Several yeast MAPs had been isolated and some of them identified [Barnes et al., 1992]. It is also a cell amenable to permeabilization providing an experimental tool for assay of intracellular enzymes in situ [Serrano et al., 1973].

We assayed the intracellular activities of HK and PK. Under those conditions, G6PDH catalyzes the conversion of G6P (the product of HK) as in vitro, and pyruvate decarboxylase and alcohol dehydrogenase catalyze the conversion of pyruvate, the product of PK. In order to test if enzymatic fluxes were responsive in situ to microtubules disassembly, we permeabilized yeast cells in the presence of nocodazole (15 μg/ml) [Jacobs et al., 1988; Solomon, 1991].

Some quantitative differences in kinetic parameters were noticed in situ with respect to the in vitro data obtained in the presence of brain MTP. In the HK/G6PDH couple nocodazole promoted a slight increase both in K_M and V_{max} respect to permeabilized control cells (Fig. 4A). PK not only retained its allosteric properties in the presence of nocodazole but also increased the in situ reaction flux respect to permeabilized control cells (Fig. 4B). Higher V_{max} values were obtained with cells treated with nocodazole (15 μg/ml) dissolved either in DMSO or ethanol (Fig. 4B). By comparing the data obtained in

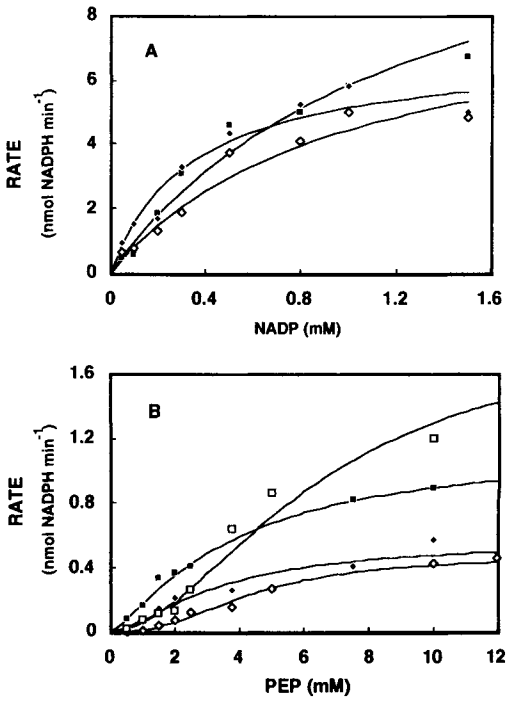

Fig. 4. Intracellular fluxes of HK and PK measured in permeabilized yeast cells in the absence (◇) or in the presence of nocodazole (■,□) or taxol (◆). *Saccharomyces cerevisiae* strain A364A yeast cells were permeabilized as described under Methods in the absence or in the presence of nocodazole (15 μg/ml) or taxol (1 μM). The permeabilized cells were used as the enzymatic source and assayed in situ essentially as described for in vitro conditions with slight differences already pointed out in Methods. The continuous lines represent the fitting to the experimental data.

vitro and in situ there is a qualitative resemblance in the kinetic behavior (mainly in V_{max}) of PK between nonpolymerized MTP and the intracellular effect of nocodazole (compare Figs. 2B, 4B). The cooperativity of PK was roughly twice lower in situ with respect to in vitro but the differences in $K_{0.5}$ were significantly larger (not shown). The explanation of the Hill coefficient differences shown by PK in vitro and in situ may be attributed in part to a predominance of monomeric-dimeric forms of the enzyme of lower activity than the tetrameric-pentameric oligomerization that apparently predominates in vitro (Table I). In the next section we develop a model by which MTP favors the stabilization of the higher oligomeric states of PK. Taxol-treated cells showed similar fluxes respect to permeabilized control cells for both enzymatic couples (Fig. 4A,B). This result is in agreement with the reported inability of taxol to stabilize microtubules assembled from pure yeast tubulin in vitro (Barnes et al., 1992).

We further assessed the effect of nocodazole in permeabilized yeast cells by immunofluorescence of tubulin containing structures (Fig. 5). Permeabilized controls (in the presence of DMSO and in the absence of the drug: see Methods) (Fig. 5A) as well as taxol-treated cells showed similar staining, sometimes as dots in the cells' periphery (not shown). Extensive disassembly of microtubules in nocodazole-treated cells was visualized as diffuse and less intense fluorescence staining (Fig. 5B). These results are in agreement with the kinetic data determined in situ (Fig. 4A,B).

Modelling HK/G6PDH and PK/LDH Fluxes and Kinetic Behavior in the Presence of Brain MTP

The dependence of the HK/G6PDH flux on MTP concentration suggested that the soluble components of MTP could account for the activation of the flux through these enzymes. The activation obtained in the presence of polymerized brain MTP reached a plateau level when the concentration of soluble MTP coexisting with the microtubular structure became steady (Fig. 1A). In addition the enzyme couple does not appear to interact strongly or permanently as could be judged from the results shown in Figure 3A. We tested the hypothesis above by modelling the enzyme fluxes as described in the Appendix. The simulation results allowed us to reproduce the experimental data shown in Figure 2A (indicated as asterisks). The test of the 0.8, 1.2, and 1.5 mg/ml MTP gave good agreement between simulated and experimental values (within 5% error). These results support our hypothesis that the MTP not engaged in the microtubule structure is activating G6PDH.

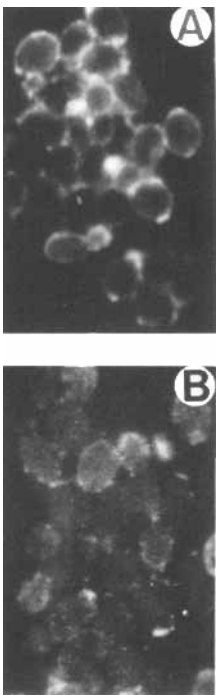

Fig. 5. Indirect antitubulin immunofluorescence micrographs of permeabilized yeast cells. *Saccharomyces cerevisiae* strain A364A yeast cells were grown, treated in the absence (**A**) or in the presence of 15 μg/ml nocodazole (**B**) and permeabilized as described under Methods. Tubulin containing structures were visualized as described before [Pringle et al., 1991; Cáceres et al., 1992].

The putative mechanism of activation of PK/LDH flux was addressed by taking into account that the average catalytic activity of an enzyme if depending on its degree of self-association, could be affected by molecular crowding if the latter alters the average degree of self-association (monomer, dimer, tetramer) [Minton and Wilf, 1981]. According to the data shown in Table I, the Hill coefficient, n, of PK increased from 3.31 ± 0.35, in the absence, to 4.48 ± 0.31 in the presence of brain MTP. Polymerized-MTP also promoted an increase in n. This change in the degree of cooperativity favors the idea that in the presence of either polymerized- or nonpolymerized-MTP the average degree of self-association increases together with the maximal activity of the enzyme. The latter is reinforced by the fact that the intracellular V_{max} values of PK, in correspondence with decreased ns, are much lower than in vitro. Taking into account the considerations above we developed a model that allowed us to account for our experimental data (see Appendixes).

The simulation results allowed us to reproduce the rate values shown in Figure 2B. In addition we were able to predict the V_{max} and n that was in fact displayed by the enzyme system at 1 mg/ml MTP. On the other hand, since no changes in the $K_{0.5}$ of PK were registered it could be inferred that the mechanism of activation by MAPs through self-association of the enzyme differs from the activation elicited by an allosteric effector, such as fructose 1,6-bisphosphate (FDP). The same activation should be observed when the experimental design of Figure 2 is carried out in the presence of FDP (Fig. 2B: left traces). The activity measured in the absence of MTP (Fig. 2B) clearly shows a 3.5-fold decrease in $K_{0.5}$ in the presence of FDP (10 μM) with respect to its absence (Fig. 2B). An increase of 1.7 times in V_{max} together with a moderate increase in n (from 4.48 ± 0.31 to 5.12 ± 0.43) were also noticed in the presence of

FDP and nonpolymerized MTP. Moreover, the intracellular PK flux was insensitive to the same concentrations of FDP used in vitro (results not shown). These results are in agreement with the effect of MTP on PK's activity being mediated by a change in its degree of association rather than an allosteric one. Similarly, the PK/LDH system was assayed in vitro in the presence of FDP for polymerized brain MTP or the absence of MTP. The results obtained were not significatively different to those obtained in the absence of FDP (Fig. 2B).

DISCUSSION

The main goal of our work is to show a concentration-dependent stimulatory effect in the presence of polymerized or nonpolymerized microtubular protein on metabolic fluxes sustained by enzymes of carbon metabolism. The regulatory pattern in the presence of polymerized or nonpolymerized brain MTP was to increase fluxes in both enzymatic couples tested (Fig. 1). We provide evidence that in permeabilized yeast cells PK, able to interact with the microtubular lattice, was sensitive to nocodazole (Figs. 3B, 4B). Furthermore, a noninteracting system such as HK/G6PDH was not sensitive to nocodazole (Figs. 3A, 4A). We further show that intracellularly, the effect of nocodazole correlated with its expected effect on the microtubular lattice (Fig. 5) [Jacobs et al., 1988; Solomon, 1991].

The stimulatory effect was shown by kinetic studies of the two coupled-enzyme assays, using MTP preparations from brain which contained 90% of protein comigrating with standard rat tubulin and exhibiting a small amount of MAPs ($\sim 10\%$) as could be judged by SDS-PAGE (not shown). The microtubular protein concentration range (1–15 μM) explored in the present work correspond to the physiological range. It has been reported that the intracellular concentration of tubulin in *Xenopus* oocytes is 2.5 μM [Kirschner and Mitchison, 1986], while in *Spisula* oocytes it is 23 μM [Suprenant, 1991]. In fact, 100 μg/ml of MTP ($\sim 90\%$ of the total protein, tubulin) corresponds to ~ 1 μM of tubulin dimer.

The regulatory pattern in the presence of polymerized brain MTP was also to increase fluxes in both enzymatic couples tested (Fig. 1). The increment in fluxes in the presence of polymerized MTP as a function of MTP concentration showed the following characteristics: (1) flux-saturation for MTP concentrations higher than 0.8 mg/ml as in the case of HK/G6PDH assay (Fig. 1A); and (2) an almost linear increase in fluxes in the low concentration range of MTP (Fig. 1A: HK/G6PDH) or for a wide range of MTP concentrations (Fig. 1B: PK/LDH).

The results obtained from the interaction experiment of the HK/G6PDH enzymatic couple indicated that the change in the regulatory properties and kinetic parameters of the enzyme upon transitions in the polymeric state of the microtubular network, did not depend on a strong or permanent interaction of the enzyme(s) with the structure. Since 70% of the protein polymerizes at MTP concentrations of 0.8 mg/ml under our experimental conditions, one could argue that the flux activation effect in the case of HK/G6PDH may be due to the soluble material after polymerization. Table II shows that a flux activation of 17% in the HK/G6PDH system exists in the case of brain tubulin polymerized in the presence of taxol (no free tubulin) and 41% activation in the presence of purified tubulin. Therefore, it appears that the maximum (60%) flux stimulation by polymerized brain MTP in this enzymatic couple (Fig. 1A) is a mixed effect of the soluble protein and the whole lattice (tubulin plus MAPs) since in the presence of purified MAPs we were unable to recover the activation effect observed (Table II). The activation effect of soluble MTP in the HK/G6PDH catalyzed flux was taken into account in the modeling (see Appendix A). We were able to reproduce the experimental data shown in Fig. 2A; likely then the activation of the HK/G6PDH flux (Fig. 1A) is mediated by soluble MTP. In permeabilized yeast cells, the HK/G6PDH system which was shown not to interact with the brain MTP lattice in vitro, exhibited in situ a kinetic behavior close to that of the enzymatic couple in the presence of polymerized brain MTP (compare Figs. 2A, 4A). The apparent insensitivity of HK/G6PDH activity to the intracellular state of microtubules as could be judged from permeabilizing cells in the presence of nocodazole (Figs. 4A, 5A,B), suggests that the enzymes "feel" already a structured intracellular environment.

The flux activation is shown to be MAPs-mediated for the enzymatic couple PK/LDH involving PK's allosteric behavior (Table II). The experiment shown in Table II also strongly suggests that the interaction of the PK/LDH enzymatic couple with the network of brain MTP is at least in part, MAPs mediated. We explained the flux activation induced by MTP in the PK/LDH couple by a displacement of the equilib-

rium of association of the allosteric enzyme by stabilization of the higher oligomeric states coincident with higher activity. With the model described in Appendix B we have been able to reproduce our experimental data (Figs. 1, 2). The simulation results together with experimental values in the presence of the allosteric activator FDP allowed to confirm that the activation effect provoked by MTP is consistent with a stabilization of higher oligomeric forms of the enzyme instead of an allosteric change (Fig. 2; Table II). That the same mechanism might be at work intracellularly, was suggested by the insensitivity in situ of PK's flux to FDP. The specificity of the effects promoted by MTP on PK/LDH flux is stressed by the fact that the activation at least in vitro is MAP-mediated. Furthermore, high concentrations of neutral polymers or actin in the same concentration range of brain MTP, did not influence or inhibited rather than stimulated the enzymatic flux (results not shown). Interestingly, the PK/LDH couple which interacts in vitro, was also sensitive in situ to the state of polymerization of microtubules. In fact, PK's behavior in yeast cells permeabilized in the presence of nocodazole changed in the same sense of increase in V_{max} as the in vitro data (compare Figs. 2B, 4B). A still higher increase registered with nocodazole dissolved in ethanol was accompanied by higher cooperativity in correspondence with the in vitro data. At present these data only allow us to infer by analogy with the in vitro data, that by changing the amount of intracellular tubulin might be possible to show flux activation as in Figure 1B. That the latter is a likely possibility is suggested by the fact that we were able from the fit of our experimental data shown in Figure 2B to predict the fluxes (which correspond to V_{max} values) measured in Figure 1B.

The activity of some enzymes like glyceraldehyde-3-phosphate dehydrogenase (GAPD) (Minton and Wilf, 1981) and PFK [Roberts and Somero, 1987] have been shown to be affected by macromolecular crowding through stabilization of certain oligomeric states of the catalysts. In the case of GAPD the addition of unrelated globular proteins promoted the formation of tetramer due to crowding or space-filling properties of the added species [Minton and Wilf, 1981]. Binding of PFK to actin appeared to stabilize the active tetrameric form of the enzyme [Roberts and Somero, 1987] which was coincident with kinetic activation [Luther and Lee, 1986]. Our results fit into this general picture and further raises the interesting possibility that under cellular conditions activation of enzymatically-catalyzed fluxes might depend on the polymerization status of MTP and its concentration (Fig. 1).

We further investigated whether the presence of brain MTP introduced changes in medium viscosity which could in turn impose diffusional restrictions to substrates or products of the enzymatic reactions. Medium viscosity was studied through the steady-state fluorescence polarization spectroscopy of the probe 2,7-bis-(2 carboxyethyl)-5-(and 6)-carboxyfluorescein (Dix and Verkman, 1990). The increase in viscosity in the presence of either non-polymerized or polymerized brain MTP was low enough to introduce diffusional limitations in our in vitro conditions for molecules of low molecular weight such as the enzymes' substrates (data not shown).

Glyceraldehyde phosphate dehydrogenase, aldolase, PK and LDH have been reported to interact with tubulin while yeast hexokinase did not [Karkhoff-Schweizer and Knull, 1987]. These data agree with the results of the interaction experiment presented here (Fig. 3). We found no evidence of interaction of the microtubular structure for the enzyme couple HK/G6PDH; the isoelectric point of G6PDH being 5.3 [Aon et al., 1989]. We did find interaction between the enzyme couple PK/LDH and microtubules; both enzymes with isoelectric points greater than 8, then positively charged at pH 6.9. At pH 6.9, tubulin carries a negative charge. This results suggests that the interaction between enzymes and cytoskeleton structures would depend on electrostatic interactions as already proposed [Karkhoff-Schweizer and Knull, 1987]. However, the experiments in the presence of polymerized actin which also carries a negative charge at pH 6.9 did show different results. Under conditions in which both enzymatic couples did not interact with polymerized actin, the fluxes were slightly inhibited through the HK/G6PDH couple, and significatively in the PK/LDH system (not shown). Therefore, the idea is reinforced that the effect of flux activation is specifically induced by the MTP lattice in itself or its components such as MAPs for PK (Table II).

Overall, the results presented suggest to us that the assembly of large supramolecular structures such as microtubules is likely to influence the behavior of enzymatic fluxes, acting as a synchronizing mechanism of cellular processes. Alterations in the regulation of pathway kinetics may imply changes in the fluxes of metabolic pathways during the cell cycle and specially in

mitosis when the mitotic spindle should be organized from microtubules [Belmont et al., 1990].

In the ground of living cells, e.g., neurons [Mitchison and Kirschner, 1988] the microtubular network could be involved in the general intracellular signal transduction pathway of environmental stimuli. The dynamics of metabolic fluxes when coupled with the autonomously self-organized properties of microtubules [Gelfand and Bershadsky, 1991; Carlier, 1992] might confer new properties to the cell through the synchronization of several simultaneously occurring cellular processes. Two important features of the spatiotemporal organization of microtubular networks make them worth candidates as a coherent synchronizer of different cellular activities: (1) their behavior as non linear, dissipative systems and therefore the potential ability to macroscopically self-organize under far from thermodynamic equilibrium conditions; and (2) their nature of an "ordered fractal," i.e., structural self-similarity at several spatial length scales [Rabouille et al., 1992].

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APPENDIX A

The HK/G6PDH model proposes that soluble microtubular protein activates the rate of the enzymatic couple through the interaction with the enzyme G6PDH. This activating interaction is modelled by an additional term in the rate equation that depends explicitly on MTP through a saturable function as follows:

$$V^0_{G6PDH} = \frac{V'_{max}[NADP][G6P]}{[NADP][G6P] + K_M^{NADP}[G6P] + K_M^{G6P}[NADP] + K_M^{NADP}K_M^{G6P}} + \frac{V''_{max}[NADP][G6P][MTP]}{[NADP][G6P][MTP] + K_M^{NADP}[G6P][MTP] + K_M^{G6P}[NADP][MTP] + K_M^{MTP}[NADP][G6P] + \cdots} \cdots + K_M^{MTP}K_M^{G6P}[NADP] + K_M^{MTP}K_M^{NADP}[G6P] + K_M^{NADP}K_M^{G6P}[MTP] + K_M^{NADP}K_M^{G6P}K_M^{MTP} \quad (1)$$

being V'_{max}, V''_{max} the maximal initial rates of G6PDH taken from experimental data in the absence or the presence of MTP. K_M^{NADP}, K_M^{G6P}, K_M^{MTP} are the Michaelis-Menten constants for NADP, G6P and soluble MTP whose numerical values were taken from data presented in this work or elsewhere [Aon et al., 1989]. In the model formulation the K_M values of (1) are identical for both kinetic terms, being different for NADP and MTP in the presence or absence of GTP, i.e., under polymerization conditions. In the presence of GTP, only the soluble MTP coexisting with the microtubular structure was taken into account in the rate calculations.

APPENDIX B

Essentially, our model proposes that the enzyme PK may exist in two forms with different degrees of association, the larger the number of associated subunits the higher the activity of the complex. According to the latter the pentamer would exhibit larger activity than the tetramer. The interaction with MTP in its polymerized or non-polymerized forms would favor the predominance of one or the other form of the enzyme. MAPs favor the association of PK subunits as pentamers which exhibit higher activity than tetramers. The extent of association as either form is reflected in the Hill coefficient, n, as follows:

$$n_{exp} = 4\, f_{tet} + 5\, f_{pent} \quad (2)$$

being n_{exp} the Hill coefficient experimentally determined and f_{tet} and f_{pent} the fraction of PK in the tetrameric or pentameric forms, respectively. On the other hand, the experimental maximal rate of conversion by PK activity is ruled by:

$$V_{max(exp)} = V_{max(tet)} f_{tet} + V_{max(pent)} f_{pent} \quad (3)$$

being $V_{max\,(exp)}$ the V_{max} experimentally measured and $V_{max\,(tet)}$ and $V_{max\,(pent)}$ the maximum velocity of the tetrameric or pentameric forms of the enzyme, respectively. This mechanism of enzyme activation by self-association is quite different from the activation produced by an allosteric effector. With respect to the latter, our model predicts that the presence of the allosteric activator FDP should not interfere with the activation elicited by MAPs, prediction that could be experimentally confirmed (see text and Fig. 2B).

The relationship between the relative amount of the enzyme in the form of a tetramer or pentamer and the state of polymerization may be derived from the following considerations: The equilibrium between the pentameric-tetrameric forms of PK is affected by the concentration of MAPs and that of GTP in the reaction medium.

$$\frac{d[Pent]}{dt} = k_2[Tet][MAPS] - k_{-2}[Pent] - k'_{-2}[Pent][GTP] = 0, \quad \text{at equilibrium} \quad (4)$$

$$\frac{[Tet][MAPS]}{[Pent]} = \frac{k_{-2} + k'_{-2}[GTP]}{k_2} \quad (5)$$

in the absence of GTP the ratio of constants in expression (5) is equal to 0.043 mg/ml and in the presence of GTP is 0.36 mg/ml; considering that 10% of MTP corresponds to MAPs.