Heterogeneous Distribution and Organization of Cytoskeletal Proteins Drive Differential Modulation of Metabolic Fluxes

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Abstract   On the basis of experimental data obtained in vitro, we propose that differential segregation of actin and tubulin in the cytoplasm may be a regulatory mechanism of metabolic fluxes. The results presented point out that the *same* enzymes may be *differentially* modulated at different locations in the cytoplasm, depending on the cytoskeletal protein present at that location, its concentration, polymeric status, or geometric arrangement. Essentially, actin or microtubular protein would exert their effect on enzymatic catalysis through displacement of the equilibrium of enzyme oligomers either to active or less active species. The latter was corroborated by mathematical modeling of the dynamic coupling between microtubular protein assembly–disassembly and pyruvate kinase activity. From these results, a precise biochemical meaning can be given to the putative linkage existing between the mechanisms by which cells rearrange their cytoplasmic architecture and the dynamics of biochemical reactions taking place concomitantly. © 1996 Wiley-Liss, Inc.

The intracellular environment is a highly crowded protein crystal [Fulton, 1982; Luby-Phelps et al., 1988] that favors homo- or hetero-associations between enzymes, proteins, or protein and organelles [Hirokawa, 1991; Clegg, 1984, 1992; Knull and Walsh, 1992]. The enzyme microenvironment of the intracellular milieu is substantially occupied or crowded [Minton, 1992; Garner and Burg, 1994] with a large variety of macromolecules and proteins. Actin and tubulin are major structural proteins in all eukaryote cells, and their distribution changes along the cell cycle. In the budding yeast *Saccharomyces*, the cell cycle-dependent patterns of microtubule and actin localization are well stud-ied [Lew and Reed, 1993; Chant, 1994]. The yeast actin cytoskeleton, in immunofluorescence light microscopy [Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Lew and Reed, 1993; Welch et al., 1994] or immunoelectron microscopy [Mulholland et al., 1994], appears as cytoplasmic cables and cortical patches asymmetrically organized along the axis of cell growth. Reported experimental data show that microtubules and actin filaments continuously assemble/disassemble along the length of the axon of cultured neurons [Hirokawa, 1991; Morfini et al., 1994].

Previously [Cortassa et al., 1994] we reported a concentration-dependent activating effect of polymerized or nonpolymerized microtubular protein on metabolic fluxes sustained by enzymes of carbon metabolism. Additionally, experimental evidence showed that pyruvate kinase was sensitive to the polymeric status of microtubular protein in permeabilized yeast cells.

In this work, we present experimental and theoretical evidence that supports the notion

that cytoplasmic spatial segregation of cytoskeleton components (actin and microtubular protein) may provide a plausible biochemical mechanism of the spatiotemporal organization of metabolism. Cellular regulation of metabolic fluxes may be achieved through distinct cytoskeletal proteins, their concentration, polymeric status (assembled or unassembled), or geometric arrangement (e.g., bundles or random polymers). The regulatory effects of cytoskeleton components on metabolic fluxes are accomplished via different targets, i.e., the local activity of enzyme(s), concentration of substrates, enzymes or effectors, or different oligomeric forms of allosteric enzymes [Roberts and Somero, 1987; Lehotzky et al., 1993; Cortassa et al., 1994]. The effect of microtubular protein polymerization–depolymerization on the dynamics of PK activity was studied by mathematical modeling.

MATERIALS AND METHODS
Microtubular Protein Preparation

Microtubular protein (MTP) was prepared from rat brain as described by Cortassa et al. [1994]. MAPs separation from tubulin was performed as described by Cortassa et al. [1994]. In the present work, the preparation of MAPs was done in the presence of 0.01% PMSF.

Polymerization of Brain Microtubular Protein

MTP polymerization was monitored by absorbance at 350 nm in a cuvette thermostated at 37°C and 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. Additional controls are described by Cortassa et al. [1994].

Enzymatic Activity Determinations In Vitro

All enzymatic 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 mixture at final concentrations of 50–2,000 μg ml^{-1}. 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. The incubation mixtures of both enzy-

matic assays were performed as described by Cortassa et al. [1994].

Enzymatic Flux Determination in the Presence of Actin

G-actin (0.25–1.0 mg ml^{-1} 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. Enzymatic activity was monitored as described above.

Protein Determination

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

Materials

G-actin (from bovine muscle) and PEG 8000 were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS
Concentration and Polymeric Status of Cytoskeleton Components Differentially Regulate Metabolic Fluxes
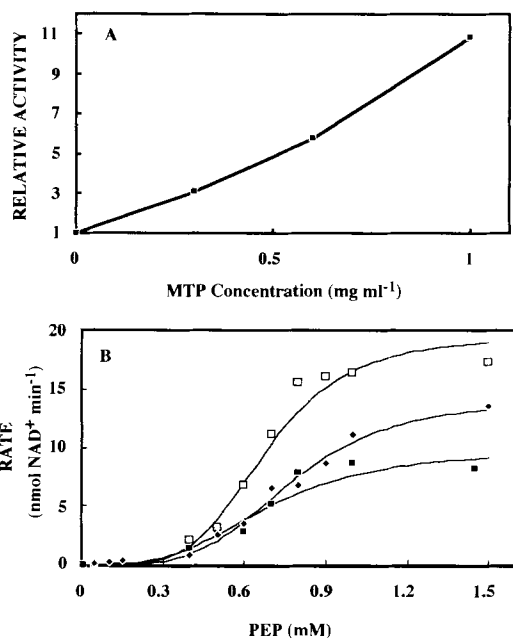
The regulatory effects exerted by microtubular protein or actin on enzyme kinetics were investigated. The enzymatic couples PK/LDH and HK/G6PDH were assayed in the presence of different concentrations of either polymerized or nonpolymerized cytoskeletal proteins.

Microtubular protein. Metabolic fluxes catalyzed by enzymes involved in carbon metabolism may be differentially modulated by the concentration and polymeric status of MTP. Fluxes were increased in the presence of different MTP concentrations (1–15 μM), either in its polymerized or in its nonpolymerized state relative to controls [Cortassa et al., 1994]. Nonpolymerized MTP increased the flux through both enzymatic couples tested (HK/G6PDH and PK/LDH). When we further improved the preparation procedure of MAPs by including protease inhibitors (0.01% PMSF), a dramatic increase in the relative activity of PK (~ 1,000%) was obtained in the presence of 1 mg ml^{-1} of nonpolymerized MTP (Fig. 1A). The flux sustained by PK/LDH increased linearly with MTP concentration. Part
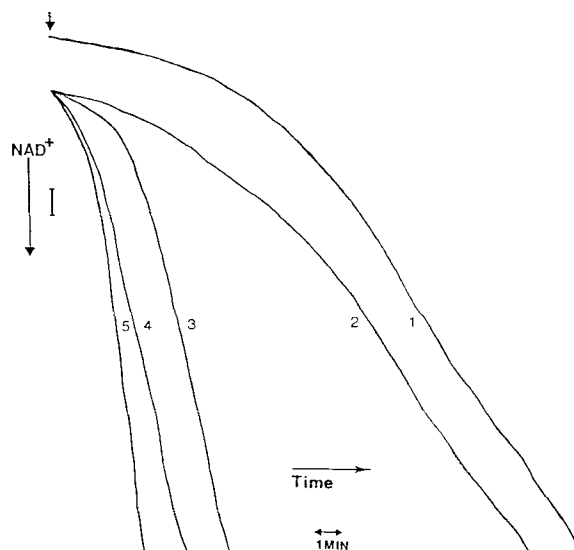
of the activity of PK/LDH could be located in the pellet of polymerized MTP [Cortassa et al., 1994].

After further purification of MAPs from tubulin, the activating effect corresponded to the MAPs fraction. Apparently, MAPs present in the preparation of MTP were able to induce the increase in flux independent of the presence of tubulin (Fig. 2, traces 2 and 4).

An insight into the mechanism of flux activation by MTP was obtained from the kinetics of the couple PK/LDH in the presence of a fixed MTP concentration in the stimulatory range (Fig. 1B). It appears that the effect of increase in fluxes was exerted mainly through V_{max} and a higher cooperativity for PK (Fig. 1B). These results were explained by modeling based on experimental evidence [Cortassa et al., 1994]. In the present work the modeling was further extended to describe quantitatively the coupling


Fig. 2. Activation of PK by MTP appears to be due to microtubule-associated proteins (MAPs). The MTP fraction was separated into tubulin and MAPs by passage through phosphocellulose as described by Cortassa et al. [1994]. Enzymatic activity was assayed as described under Materials and Methods. Plotted are the time courses of NADH absorbance recorded in a Gilford spectrophotometer as described under Materials and Methods. The activation effect is due to the (total) MAPs fraction (trace 4: 0.11 mg protein ml^{-1}) and not to tubulin (trace 2: 0.6 mg protein ml^{-1}). Trace 1 shows the control activity without either MTP or MAPs. The MTP concentration assayed in traces 3 and 5 were 0.3 and 0.6 mg protein ml^{-1}, respectively.

Fig. 1. Increase of PK activity (A) and effect on kinetic parameters (B) as a function of polymerized or nonpolymerized microtubular protein (MTP). A: Activity of the enzyme couple PK/LDH is plotted as a function of the nonpolymerized MTP concentration respect to its activity in the absence of MTP. Activity measurements in the presence of nonpolymerized MTP were assayed as described in Materials and Methods. B: In the y-axis, the initial rates of enzymatic fluxes in the absence of MTP (■) or presence of polymerized (◆) or nonpolymerized MTP (□) (0.4 mg ml^{-1} of MTP) are plotted as a function of PEP concentration. Unlike the experiments shown in A, the MTP batch employed in B was obtained in the absence of 0.01% PMSF. The continuous lines represent the fitting to the experimental data, as described by Cortassa et al. [1994].

between microtubule dynamics and PK kinetics (see section below on: *Modeling the Dynamics . . .*).

Actin. F-actin polymerized in the presence of 5% PEG exerted an opposite effect on enzyme activity with respect to that observed with MTP. HK/G6PDH and PK/LDH were inhibited to a different extent by increasing concentrations of actin (Fig. 3A). Actin, slightly inhibited (10%) the flux through the HK/G6PDH couple and significantly ($\sim$40%) at $\geq$0.5 mg ml^{-1} of PK/LDH (Fig. 3A). The putative effect of molecular crowding induced by PEG may be ruled out since controls were run in parallel in the presence of 5% PEG but in the absence of actin.

Unlike the results obtained with polymerized MTP [Cortassa et al., 1994], none of the two enzymatic couples tested was able to interact strongly with the actin lattice. After 2-h centrifugation at 40,000g, the whole activity was recovered in the supernatants (not shown).

The kinetics of PK/LDH showed differences, depending on the presence of inhibitory concentrations of F-actin (0.4 mg ml^{-1}) with different geometric arrangements induced by 7% PEG
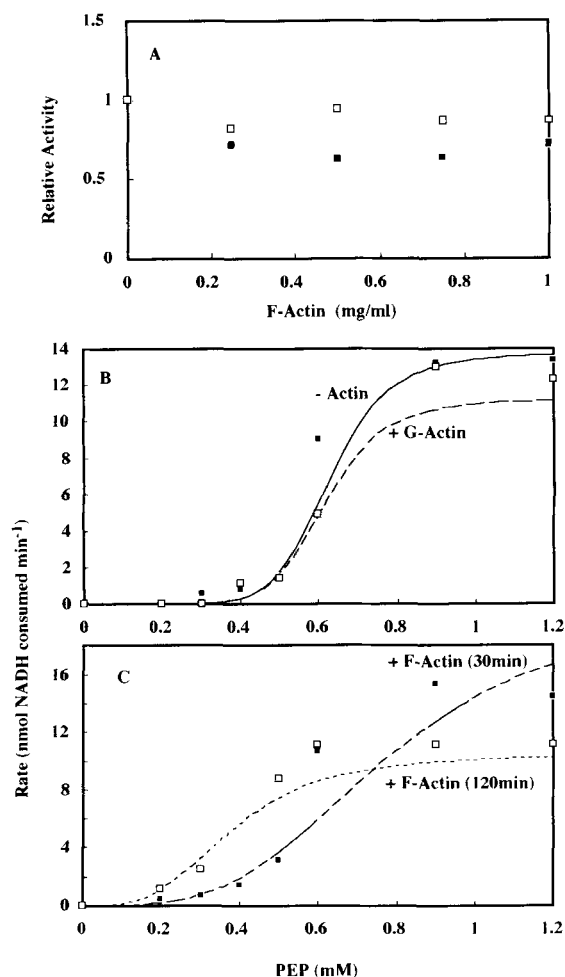
Fig. 3. Effect of F-actin and G-actin on PK/LDH and HK/G6PDH sustained fluxes and kinetic parameters. **A:** PK/LDH (■) and HK/G6PDH (□) relative activities at increasing F-actin concentrations. **B,C:** PK activity (initial rate) as a function of PEP concentration, assayed in the presence of G-actin (**B**) or F-actin (**C**) polymerized for 30 min or 120 min in the presence of 7% PEG. Actin (0.25–1.0 mg ml⁻¹ (**A**) or 0.4 mg ml⁻¹ (**C**), was polymerized as described under Methods, 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. Both polymerization and enzymatic activity were monitored as described in Materials and Methods. The G-actin concentration (**B**) corresponds to 0.4 mg ml⁻¹. The lines drawn under the experimental points are the best-fit curves according to a generalized Hill equation for the PK/LDH activity (**B,C**). The fitting procedure was performed according to the kinetic data given in Table I as described by Cortassa et al. [1994].

(Fig. 3C). Depending on the polymerization time, either random filaments (30 min) or bundles (120 min) of F-actin are induced in the presence of 7% PEG [Suzuki et al., 1989]. A drastic decrease in cooperativity with respect to the control in the absence of actin, was observed in the

presence of both actin arrangements (from n = 8.2 ± 0.3 to 3.3 ± 0.3) along with almost unchanged $K_{0.5}$ for random actin filaments ($K_{0.5}$ = 0.78 ± 0.27) and half $K_{0.5}$ (=0.38 ± 0.02) for actin bundles (Fig. 3C; Table I). Also a drastic difference was registered in V_{max} values between the two actin arrangements (20.6 ± 3.0 respect to 10.5 ± 0.8 nmol min⁻¹ in random or bundle filaments, respectively) (Fig. 3C; Table I).

Nonpolymerized G-actin behaved similarly as the control in the absence of actin (Fig. 3B). The degree of cooperativity, n, and the $K_{0.5}$ of the reaction were around 8 and 0.6 mM in both cases, whereas the V_{max} values were 11.2 ± 1.5 and 13.7 ± 1.8 nmol min⁻¹, respectively (Table I). The high cooperativity exhibited by PK in the absence or presence of G-actin was likely induced by 7% PEG, since our previous data in the absence of PEG gave n values of 3.3 [Cortassa et al., 1994].

The results presented indicate that the inhibitory effect (~35% at 0.4 mg ml⁻¹ of F-actin) shown in Figure 3A is due to a decrease of the V_{max} of PK. This is likely due to an arrangement of F-actin in bundles. In fact, a value of V_{max} ~25% lower (10.5 compared to 13.7 in Table I) was measured in the presence of actin bundles respect to the control. This explains the apparent discrepancy introduced by the increase of V_{max} values observed in the presence of random filaments of actin (20.6 with respect to 13.7 in Table I).

Modeling the Dynamics of Microtubular Protein Polymerization–Depolymerization and Enzymatic Activity

We asked whether the intrinsic dynamics of microtubules polymerization–depolymerization may be affecting in turn the dynamics of enzymatic reactions. Intuitively, one may imagine an affirmative response to this question. However, is there a plausible biochemical mechanism that explains such a coupling? A model that couples MTP dynamics and the activity of PK was developed according to Figure 4. A mathematical description of the model depicted in Figure 4 is given in the Appendix.

The model simulated the kinetics of MTP polymerization when incubated at 37°C in the presence of 1 mM GTP and different initial concentrations of microtubular protein (Fig. 5A). The effect of polymerized MTP on the increase in fluxes sustained by PK (described by Cortassa

TABLE I. Effect of G-Actin or F-Actin (0.4 mg ml^{-1}) on PK/LDH Kinetic Parameters*

	−Actin	+G-actin	+F-actin (30 min)	+F-actin (120 min)
PK/LDH				
V_{max} (nmol min^{-1})	13.7 ± 1.8	11.2 ± 1.5	20.6 ± 3.0	10.5 ± 0.8
$K_{0.5}$ (mM)	0.63 ± 0.03	0.62 ± 0.03	0.78 ± 0.27	0.38 ± 0.08
n	8.3 ± 0.3	8.2 ± 0.3	3.4 ± 0.3	3.3 ± 0.3

*The data presented in Figure 3 were fitted with a generalized Hill equation and a Levenberg-Marquardt algorithm for nonlinear regression analysis as described by Cortassa et al. [1994].
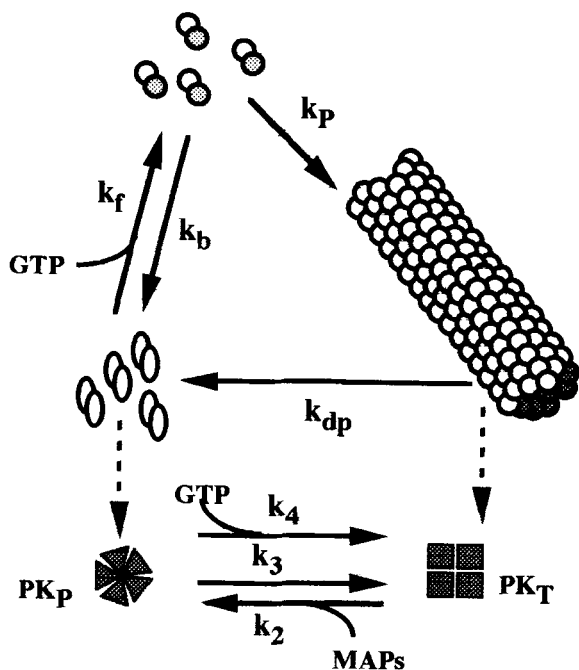From Aon et al. [1995].

Fig. 4. Scheme of the model that links microtubular protein polymerization–depolymerization dynamics and pyruvate kinase (PK) kinetics. PK in the presence of MTP, may exist as a tetramer (PK$_T$) or pentamer (PK$_P$), the proportion of each form, depending on the polymeric status of MTP, MAPs, or GTP (k$_4$, GTP). The MAPs bound to the microtubular lattice appear to promote the formation of the pentamer (k$_2$, MAPs). The cycle of MTP polymerization–depolymerization assumes that tubulin may exist in one of three forms, i.e., either polymerized or nonpolymerized, bound to GTP, or bound to GDP. The coupling between the kinetic behavior of PK and the polymeric status of MTP is realized through the term of pentamer formation, PK$_P$, which is proportional to the amount of polymerized MTP, C$_P$ in eqns. 1 and 4. Dashed arrows, polymerized or depolymerized forms of MTP stabilize either the tetrameric or pentameric forms of PK.

et al. [1994]) was satisfactorily simulated (Fig. 5B). The experimental results simulated in Figure 5 have been obtained under batch conditions. Bistability was observed when we studied the steady-state behavior of the model as a function of the rate of polymerization–depolymerization of microtubules, k$_{pol}$, i.e., two possible steady states for the same value of k$_{pol}$ [Aon et al., 1995b]. Since the subsystem that describes the dynamics of MTP assembly–disassembly displays bistability when isolated, whereas the enzyme dynamics was only monostable, we concluded that it is the dynamics of MTP that entrains the enzyme kinetics [Aon et al., 1995b].

DISCUSSION

On the basis of experimental data obtained in vitro, intracellularly, and by mathematical modeling, in this and previous work, we propose that differential segregation of actin and tubulin in the cytoplasm may be a potential regulatory mechanism of metabolic fluxes. The results presented point out that the *same* enzymes may be *differentially* modulated at different locations in the cytoplasm. Modulation may be achieved through the cytoskeletal protein (and their associated proteins) present at that location (Figs. 1–3), its concentration (Figs. 1, 3), polymeric status (Figs. 1, 3), or geometric arrangement (Fig. 3). The kinetics of enzymatic reactions measured in vitro showed sensitivity to the concentration and polymeric status of MTP or actin (Figs. 1, 3) [Cortassa et al., 1994]. The effects on enzyme kinetics exerted by both cytoskeletal polymers were opposite (compare Figs. 1A, 3A).

The sensitivity of PK kinetics to actin organization as random polymers or bundles (Fig. 3C) suggests that enzyme-catalyzed metabolic fluxes may also be regulated by the arrangement of cytoskeletal proteins. Apparently, the presence of F-actin in either of the two geometries was able to displace the higher oligomeric states of PK to lower oligomeric forms (Fig. 3B,C). The latter may be judged from the degree of cooperativity, n, achieved by the enzyme in the absence or in the presence of F-actin (Table I). Arrays of actin in bundles are likely to occur in the cytoplasm, since the abundance of proteins should be able to have a similar effect to that observed in vitro [Suzuki et al., 1989]. In vitro simulation
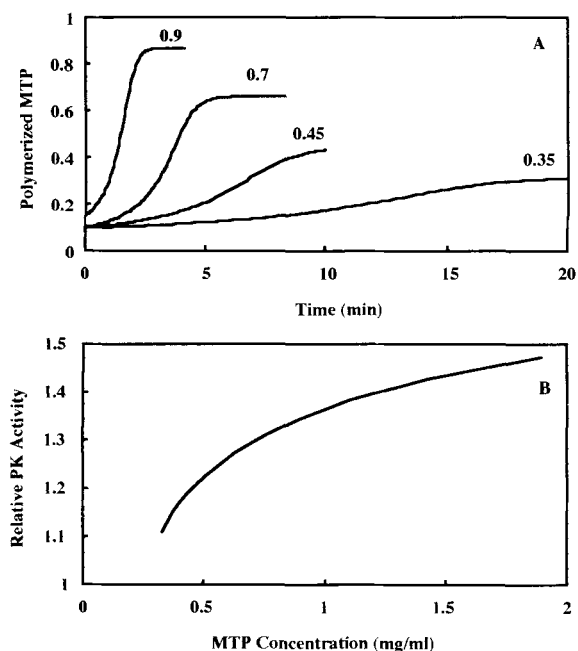
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Fig. 5. Simulations performed with the model which couples MTP dynamics and enzymatic activity. The behavior of the model depicted by ordinary differential equations (ODEs 1–4) was simulated. A: Results of the simulations of MTP polymerization kinetics at 37°C in the presence of 1 mM GTP. B: Simulation of the results obtained with the increase in the PK-catalyzed fluxes in the presence of polymerized MTP as described by Cortassa et al. [1994]. The values plotted correspond to V_{PK} (eq. 5). The equations were numerically integrated with the following parameter values: $V_T^{max} = 14$ (mM s^{-1}); $V_P^{max} = 25$ (mM s^{-1}); $K_s^n = 0.6$ (mM^n); $k_{Pol} = 10$ (mM^{-2} s^{-1}); $k_{dp} = 2.5\ 10^{-3}$ (s^{-1}); $k_f = 3.0$ (mM^{-1} s^{-1}); $k_b = 2.5\ 10^{-3}$ (mM^{-1} s^{-1}); $k_1 = 1.0$ (s^{-1}); $k_2 = 10.0$ (mM^{-1} s^{-1}); $k_3 = 0.05$ (s^{-1}); $k_4 = 0.02$ (mM^{-1} s^{-1}); PEP$_o = 10$ (mM); GTPo $= 1.0$ (mM); PK$_t = 0.01$ (mM); $n1 = 2$; Co corresponds to the values indicated on top of each polymerization curve (A) and the x-axis (B). Note that V_{max} values of tetrameric and pentameric forms of PK were obtained from the fitting of the experimental data in the absence of PMSF.

of molecular crowding with PEG or other proteins such as ovalbumin, caldesmon, and tropomyosin, induced parallel bundling of actin filaments [Suzuki et al., 1989; Cuneo et al., 1992].

More experimental work will be needed to substantiate further our proposal of cytoskeletal proteins segregation and organization as a regulatory device operating intracellularly. However, we have ascertained the plausibility of the regulatory role of cytoskeleton components by measuring in permeabilized yeast cells the same metabolic fluxes assayed in vitro. Previous work [Cortassa et al., 1994] showed that enzymatic fluxes were responsive in situ to microtubule disassembly by nocodazole [Jacobs et al., 1988; Solomon, 1991] in permeabilized yeast cells. The

effect of nocodazole promoted the increase of the in situ flux of PK that retained its allosteric properties [Cortassa et al., 1994]. By comparing the data obtained in vitro and in situ, there was a qualitative resemblance in the kinetic behavior shown by PK in the presence of nonpolymerized MTP and its intracellular equivalent in the presence of nocodazole. Essentially, PK kinetics changed in the same sense of increase in V_{max} as the in vitro data. In the HK/G6PDH couple, a slight increase both in K_M and in V_{max} was induced by nocodazole with respect to permeabilized control cells. Compounds such as taxol or cytochalasin B, which are known to stabilize microtubules or to depolymerize actin in mammalian cells, but not in yeast cells [Solomon, 1991; Barnes et al., 1992; Cortassa et al., 1994], did not affect the kinetics of either PK or HK in permeabilized yeast cells with respect to the controls (results not shown). The latter provides evidence for the reliability of our intracellular measurements of enzymatic activities, principally with respect to unspecific effects.

Hypothetically, the cytoplasm of living cells has been characterized as a random fractal of the sort of percolation clusters [Aon and Cortassa, 1994; Cortassa and Aon, 1994]. Percolation provides an explanation about the way in which cells give a coherent answer to different stimuli (e.g., hormones, neurotransmitters, substrates). Among those coherent responses, cells must divide, differentiate, migrate, or redirect metabolic fluxes [Aon et al., 1995a]. In this conceptual framework, the following question may be formulated: what consequences could entrain for enzymatic catalysis the cytoplasmic segregation of polymers that arrange according to different fractal dimensions D? An answer to this question may be given from the property of percolation clusters to connect far distant regions (global connectedness) when the occupancy of the lattice is larger than the percolation threshold, p_c [Feder, 1988; Rabouille et al., 1992; Aon and Cortassa, 1994]. Thus, in addition to the specific regulatory effects elicited by the segregation of cytoskeletal polymers, the dynamics of an enzyme reaction may extend to the whole cellular field, if it occurs in the percolation cluster, i.e., farther from p_c. By regulating its cytoskeletal protein distribution and polymeric status, the cell modulates the geometry of the lattice, and through it the percolation threshold, p_c.

Overall, our results suggest a plausible mechanism of modulation of metabolic fluxes by segregation of cytoskeletal polymers. We emphasize the cytoarchitecture rather than catalysis. In fact, the supramolecular organization of cellular architecture may in turn entrain the dynamics of enzymatic reactions through mechanisms of association–dissociation of enzymes toward higher or lower oligomeric forms with higher or lower activities (Fig. 4). The kinetic properties of PFK seem to be regulated by interaction of the enzyme with filamentous actin [Roberts and Somero, 1987] or purified tubulin [Lehotzky et al., 1993] through displacement of the equilibrium of PFK oligomers. Our modeling results allude to the fact that those equilibrium displacements might well be a main mechanism through which structural polymers exert their modulatory role.

ACKNOWLEDGMENTS

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APPENDIX

A model linking microtubular protein polymer-ization–depolymerization dynamics and enzyme kinetics. The coupling between MTP dynamics and PK activity (Fig. 4) was modeled through a system of four ODEs (Eq. 1–4). The equations which describe the activity of PK (Eq. 5–7) were obtained by fitting the kinetic behavior of the enzyme with respect to the concentration of its substrate phosphoenolpyruvate (PEP), in the presence of either polymerized or nonpolymerized MTP. Also the dependence of PK activity as a function of MTP concentration was taken into account [see Cortassa et al., 1994]. PK was modeled as an allosteric enzyme whose rate equation followed a Hill equation (eq. 5; see also legend to Fig. 3). The enzyme, in the presence of MTP, may exist as a tetramer (T) or pentamer (P); the proportion of each form depending on the polymeric status of MTP (Fig. 4). The MAPs bound to the microtubular lattice appears to promote the formation of the P form [see Cortassa et al., 1994 and Fig. 1B]; the V_{PK}^{max} of the P form being larger than that of the T one (Eq. 6). Indeed, the Hill coefficient, n, also changes with the degree of self-association of the enzyme (Eq. 7). Coupling with the polymeric status of MTP is realized through the term of pentamer formation in Eq. 6, which is proportional to the amount of polymerized MTP, Cp. 0.1 is the proportionality constant that accounts for the fact that MAPs represents a 10% of total MTP (Eq. 1) [see Cortassa et al., 1994].

The equations that describe the MTP polymer-ization–depolymerization assume that tubulin may exist in one of three forms, i.e., either polymerized (C_P), or nonpolymerized (C_T), bound to GTP; or nonpolymerized bound to GDP (C_D) [Carlier et al., 1987]. A conservation equation relates the three forms of MTP (Eq. 8):

$$\frac{d[P]}{dt} = 0.1k_2(PK_t - [P])Cp \tag{1}$$

$$- k_3[P] - k_4[P][GTP]$$

$$\frac{d[PEP]}{dt} = -V_{PK} - k_1(PEP_o - [PEP]) \tag{2}$$

$$\frac{dC_T}{dt} = -k_{Pol}C_P^{nl}C_T + k_fC_D[GTP] \tag{3}$$

$$- k_bC_T(GTP_o - [GTP])$$

$$\frac{dC_P}{dt} = k_{Pol}C_P^{nl}C_T - k_{dp}C_P \tag{4}$$

$$V_{PK} = \frac{V_{PK}^{max}[PEP]^n}{K_s^n + [PEP]^n} \tag{5}$$

$$V_{PK}^{max} = V_T^{max} + (V_P^{max} - V_T^{max})\frac{[P]}{PK_t} \tag{6}$$

$$n = 4 + \frac{[P]}{PK_t} \tag{7}$$

$$C_o = C_D + C_T + C_P \tag{8}$$