2-Deoxyglucose and Cytochalasin D Modulate Aldolase Mobility in Living 3T3 Cells

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Abstract. Approximately 23% of the glycolytic enzyme aldolase in the perinuclear region of Swiss 3T3 cells is immobile as measured by FRAP. Previous studies suggest that the immobile fraction may be associated with the actin cytoskeleton (Pagliaro, L. and D. L. Taylor. 1988. *J. Cell Biol.* 107:981–991), and it has been proposed that the association of some glycolytic enzymes with the cytoskeleton could have functional significance, perhaps involving a fundamental relationship between glycolysis, cytoplasmic organization, and cell motility. We have tested the effect of a key glycolytic inhibitor and an actin cytoskeletal modulator on the mobility of aldolase in living cells

directly, using fluorescent analog cytochemistry and FRAP. We report here that the competitive hexokinase inhibitor 2-deoxyglucose releases the bound fraction of aldolase in 3T3 cells within 10 min, and that this process is reversible upon washout of the inhibitor. A similar result is produced with the actin-binding agent, cytochalasin D. These results are consistent with models in which glycolytic enzymes are not exclusively diffusion-limited, soluble proteins, but may exist partially in the solid phase of cytoplasm. Such organization has significant implications for both the modulation of cytoplasmic structure and for cellular metabolism.

OUR understanding of cellular organization and function has evolved largely within the conceptual framework of studying either the structure of fixed cells or the activity of biochemical processes in relatively dilute, aqueous solutions. These approaches have been enormously valuable in elucidating the anatomy and possible biochemical function of cells and their organelles, but the dynamics of many cellular processes remain poorly understood, due to the loss of temporal information in ultrastructural studies, and to the spatial averaging inherent in biochemical experiments. Because of this, we believe that biochemical pathways must also be studied in living cells. Such experiments may reveal dynamic, but metabolically important, molecular associations which result from weak interactions at high intracellular molar concentrations. Consequently, studying the temporal and spatial dynamics of chemical and molecular processes in living cells is essential for extending our understanding of cellular functions (Taylor and Wang, 1980; Taylor et al., 1984).

We have recently mapped the distribution and mobility of two glycolytic enzymes in vivo as a model system for studying the intracellular behavior of a biochemical pathway that is traditionally characterized as existing entirely in the soluble phase. Using fluorescent analog cytochemistry and digital imaging microscopy, we presented evidence that aldolase, which bound to F-actin in vitro, was relatively concentrated in a microdomain around stress fibers in vivo (Pagliaro and Taylor, 1988), while enolase, which had no actin-binding ac-

tivity in vitro, did not exhibit stress fiber localization in vivo (Pagliaro et al., 1989). Additionally, FRAP measurements of the mobility and diffusion coefficient of the fluorescent analogs revealed that aldolase had a significant immobile fraction in vivo, while enolase did not, again consistent with in vitro FRAP experiments. The bound fraction of aldolase is released from F-actin in vitro when fructose-1,6-bisphosphate (the substrate for aldolase) is added to gelled aldolase-F-actin mixtures. Our data were consistent with models in which some glycolytic enzymes partition preferentially in microdomains around cytoskeletal elements (Clegg, 1984; Masters, 1984), or proposals that some glycolytic enzymes might exhibit 'functional duality,' in which they possess both structural and catalytic roles (Clarke et al., 1985a). Functional duality may contribute to the formation and regulation of cytoskeletal structures; organization of glycolytic enzymes around the cytoskeleton could, in turn, have significant implications for metabolic regulation and cell motility (Clarke et al., 1985b).

There is growing evidence that glycolytic metabolism is closely associated with cell motility and cytoskeletal organization. In a series of elegant experiments in the 1950s, Kamiya and colleagues studied the rhythmic "shuttle-streaming" motility of the plasmodium of the acellular slime mold *Physarum*. They found that inhibitors of glycolytic metabolism (including iodoacetate and sodium fluoride) reversibly decreased the motive force of shuttle-streaming by 75% within 2–3 min, but that respiratory inhibitors (including

2,4-dinitrophenol and cyanide) had a much smaller effect only after 20 min or more (Kamiya et al., 1957). In 1976, Michl and colleagues showed that 2-deoxyglucose (2-DG)[1] selectively inhibited receptor-mediated phagocytosis in mouse peritoneal macrophages (Michl et al., 1976a), and that the inhibitory effects of 2-DG on phagocytosis were uncoupled from bulk ATP levels in these cells (Michl et al., 1976b). Premature ischemic contracture due to ATP depletion in rat hearts has been shown to be more sensitive to glycolytic inhibitors (iodoacetate and 2-DG) than to respiratory inhibition with atractyoloside (Bricknell et al., 1981). In a study on rat epithelial cells, Gibbins showed that 2-DG stopped cell motility within 1–2 min, but that cellular ATP was not significantly depleted for more than 20 min (Gibbins, 1982). Finally, it has been shown that cellular ATP levels decrease by 25% within 1 min after perfusion of Xenopus tadpole heart endothelial cells with 2 µg/ml (∼4 µM) cytochalasin D (CD), and that the ATP decrease is not due to reduced respiration (Tillmann and Bereiter-Hahn, 1986). These lines of evidence provide substantial support for the concept that the ATP source for some kinds of cell motility is preferentially glycolysis, rather than respiration. Further, glycolytic activity may be modulated by reversible associations with the solid phase of cytoplasm, including the actin-based cytoskeleton.

Based on the above work, we hypothesized that if some glycolytic enzymes are preferentially localized around the actin cytoskeleton, and if such cytoskeletal localization is necessary for preferential use of glycolytic ATP for cell motility, then agents which inhibit glycolysis and cell motility might also affect enzyme localization. Depletion of 'downstream' glycolytic intermediates by 2-DG could decrease catalytic activity and significantly affect enzyme–enzyme and (if they exist) enzyme–structural protein interactions in vivo. The simplest prediction based on our hypothesis is that inhibition of glycolysis with 2-DG would result in the loss of the immobile fraction of aldolase in vivo, but that enolase would be relatively unaffected by 2-DG. We have tested that prediction directly by performing FRAP experiments, both before and after perfusion with medium containing 2-DG, in individual living cells microinjected with fluorescent analogs of aldolase (rhodamine-labeled aldolase, Rh-aldolase) and enolase (fluorescein-labeled enolase, Fl-enolase). To directly evaluate the role the actin cytoskeleton played in maintaining the immobile fraction of Rh-aldolase in cells, we also performed FRAP experiments on cells before and after perfusion with CD.

Materials and Methods

Cell Culture

Swiss 3T3 fibroblasts (CCL92, American Type Culture Collection, Rockville, MD; passage numbers 120–131) were cultured in DME (Sigma Chemical Co., St. Louis, MO) on 40-mm-round no. 1.5 coverslips (Erie Scientific, Erie, PA) in 60-mm tissue culture dishes essentially as described previously (De Biasio et al., 1987). To obtain cell polarity and spreading optimal for spatially resolved photobleaching experiments, cells were "starved" in DME containing 0.2% FCS (Gibco Laboratories, Grand Is-

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1. Abbreviations used in this paper: CD, cytochalasin D, D_{cyto}, apparent cytoplasmic diffusion coefficient; Fl-enolase, fluorescein-labeled enolase; Rh-aldolase, rhodamine-labeled aldolase; 2-DG, 2-deoxyglucose.

land, NY) beginning ∼25 h before microinjection with fluorescent analog(s) at the start of an experiment. The medium in the dishes containing the coverslips was exchanged for fresh, pH equilibrated DME containing 10% FCS 30–45 min before microinjection with fluorescent analog(s). Immediately after microinjection, the medium was again gently exchanged with fresh DME + 10% FCS. Cells were maintained at 37°C during microinjection. During photobleaching experiments, cells were cultured in a modified (Bright et al., 1987) Sykes-Moore chamber (Bellco Glass, Inc., Vineland, NY) mounted on a thermoelectrically controlled stage (Rainin Instrument Company, Inc., Woburn, MA). Additional temperature control was provided by an air curtain (Sage Instruments, Cambridge, MA) regulated by a proportional controller (Cole-Parmer Instrument Company, Chicago, IL), which maintained the temperature of the microscope stage and objective at 37°C.

Fluorescent Analog Cytochemistry

Fluorescent analogs of aldolase (Rh-aldolase) and enolase (Fl-enolase) were prepared and evaluated for biological activity as described previously (Pagliaro and Taylor, 1988; Pagliaro et al., 1989). For these experiments, aldolase was labeled with 5-(and-6)-carboxytetramethyl-rhodamine succinimidyl ester (Molecular Probes, Inc., Eugene, OR), and enolase was labeled with 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes, Inc.). Cells were microinjected with ∼5–10% of a cell volume to a level of 2–4% of endogenous cellular aldolase or enolase, respectively. The injection buffer consisted of 1 mM Pipes, 1 mM MgCl, 50 mM KCl, pH 7.3.

Fluorescence Redistribution After Photobleaching

FRAP experiments were performed as described previously (Pagliaro and Taylor, 1988; Pagliaro et al., 1989). For experiments in which Fl-enolase and Rh-aldolase were comicroinjected into cells; an argon ion laser (Spectra Physics, Inc., Mountain View, CA) was re-tuned between the 488- and 514-nm lines for successive FRAP measurements, and the epifluorescence filters were changed accordingly.

Inhibitor Experiments and Perfusion

All cell perfusions were performed using a peristaltic pump to regulate the flow of 37°C, CO_2-equilibrated medium to the inlet port of the modified Sykes-Moore chamber on the microscope stage. Perfusions were performed at a rate sufficient to provide a complete change of medium in the chamber and all associated tubing in 30 s (∼2.5 ml/min); at this rate fluid shear in the chamber did not produce noticeable artifacts. 2-DG (Sigma Chemical Co.) was substituted for the glucose in DME (1 mg/ml) during preparation of otherwise identical batches of medium, and 2-DG–DME was perfused into the chamber for 2-DG experiments. CD (Sigma Chemical Co.) was dissolved in a 1.0 mg/ml stock solution in DMSO (Sigma Chemical Co.), and diluted to a final concentration of 0.5 µM (253 ng/ml) in DME for CD experiments. In some experiments, the medium containing inhibitor (2-DG or CD) was washed out of the cell chamber with several changes of fresh, CO_2-equilibrated medium over a period of ∼90 s, to evaluate the reversibility of inhibitor effects on Rh-aldolase mobility.

Results

2-DG Releases the Immobile Fraction of Aldolase In Vivo

Perfusion of medium containing 1 mg/ml 2-DG into the cell chamber rapidly released the ∼21% bound fraction of Rh-aldolase in the perinuclear region of 3T3 cells, while the mobility of Rh-aldolase in the cell periphery, and the mobility of Fl-enolase, were not significantly affected. The data in Table I represent FRAP measurements taken 10–15 min after perfusion of 2-DG–DME into the cell chamber. By 10 min after perfusion, the response of cells to 2-DG was relatively uniform; in the first few minutes after perfusion, the response was heterogeneous, with some "rapid responders" (60–90 s) and some "slow responders" (90 s–10 min). The apparent cytoplasmic diffusion coefficient (D_{cyto}) of Rh-aldo-

Table I. In Vivo FRAP Data: Effect of 1 mg/ml 2-DG on Aldolase and Enolase Mobility

	Control		+ 2-DG	
	$D_{37},\text{cyto} \times 10^8$*	% Mobile	$D_{37},\text{cyto} \times 10^8$	% Mobile
Rh-Aldolase				
Perinuclear (8)‡	14.3 ± 6.7§	79.9 ± 7.7	9.11 ± 2.3	105.1 ± 6.1
Peripheral (7)	8.10 ± 3.2	101.8 ± 7.8	6.69 ± 3.9	101.8 ± 6.6
Fl-Enolase				
Perinuclear (3)	12.4 ± 9.0	98.3 ± 8.1	10.3 ± 4.1	102.4 ± 1.3
Peripheral (3)	9.63 ± 4.6	100.9 ± 3.3	7.61 ± 1.6	98.3 ± 5.1

* Apparent cytoplasmic diffusion coefficient (cm^2/s) measured at 37°C.
‡ Number of measurements used to calculate the mean.
§ Mean ± the sample standard deviation.

lase and Fl-enolase, both in the perinuclear region and the cell periphery, decreased slightly. No significant morphological changes were evident in these cells after 2-DG perfusion. In washout experiments, 80–90% of the control perinuclear bound fraction of Rh-aldolase returned by 60 min after washout (Fig. 1), indicating that the effects of 2-DG were largely reversible, but the reversal was considerably slower than the initial effect.

Cytochalasin D Releases the Immobile Fraction of Aldolase In Vivo

We used CD to study the effect of disrupting the actin cytoskeleton on the mobility of the bound fraction of aldolase. Perfusion of medium containing 0.5 μM CD into the cell chamber caused a rapid and complete release of the bound fraction of Rh-aldolase. Fig. 2 *B* shows the mobility of Rh-aldolase and Fl-enolase in the perinuclear region of two different representative cells after perfusion with CD and subsequent washout. Four other experiments yielded similar results (data not shown). In the example shown, the immobile fraction of Rh-aldolase decreased from 25.5% before CD perfusion to 2.3% by 90 s after perfusion. When CD was subsequently washed out of the chamber, 80–90% of the control perinuclear immobile fraction of Rh-aldolase returned after 20 min. Fl-enolase was 100% mobile, and its
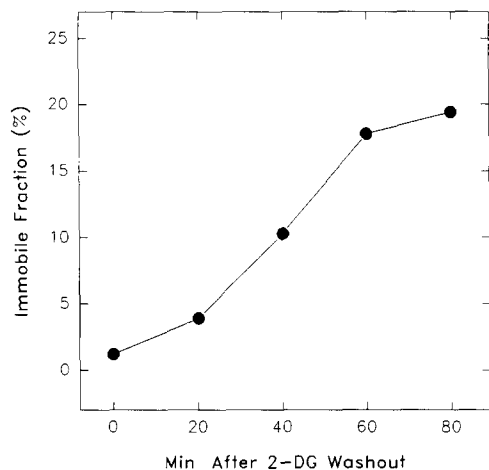
mobility was not significantly affected by either CD perfusion or subsequent washout. The D_{cyto} values of Rh-aldolase and Fl-enolase were not significantly affected by CD perfusion (Fig. 2 *A*). The measured D_{cyto} of Fl-enolase (90,000 mol wt) was slightly higher than the D_{cyto} of Rh-aldolase (160,000 mol wt), as expected, due to the larger hydrodynamic radius of aldolase.

Discussion

Inhibition of Glycolysis, Enzyme Mobility, and the Actin Cytoskeleton
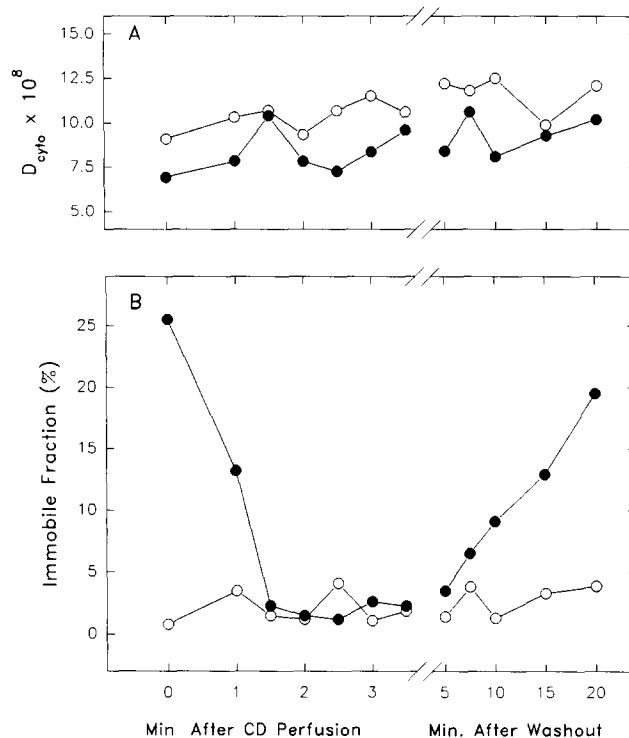
Our data demonstrate that 2-DG reversibly releases the

Figure 2. Effect of CD perfusion and washout on the mobility and D_{cyto} of Rh-aldolase (●) and Fl-enolase (○) in the perinuclear region of representative cells. FRAP was used to measure the D_{cyto} (*A*) and mobile fraction (*B*). Times after perfusion of the cell chamber with medium containing CD, and after subsequent washout are shown. D_{cyto} values are expressed in $cm^2/s \times 10^8$.

Figure 1. Effect of 2-DG washout on the perinuclear immobile fraction of Rh-aldolase in a typical cell. Timescale represents minutes after CD washout.

bound fraction of Rh-aldolase in the perinuclear region of living Swiss 3T3 fibroblasts within 10 min after perfusion, but that it does not significantly affect the mobility of Fl-enolase. This finding is consistent with models in which some glycolytic enzymes are associated with the actin cytoskeleton in an activity-dependent manner (Wilson, 1988). The reversal of the effects of 2-DG on aldolase mobility was considerably slower and somewhat less complete than the initial effect, but it was substantial and reproducible.

CD inhibits elongation of actin filaments at the barbed end and binds to actin monomers, promoting dimer formation and ATP hydrolysis (Goddette and Frieden, 1986; Cooper, 1987). CD probably changes the critical concentration for actin polymerization in vivo, and may affect cytoplasmic ATP concentrations. There is evidence that CD decreases the mean length of actin filaments in vivo, effecting a solation and contraction of the actin-myosin II system (Kolega et al., 1991), which results in fragmenting and altered organization of the actin cytoskeleton (Schliwa, 1982). Our experiments with CD demonstrated that it resulted in rapid mobilization of the bound fraction of Rh-aldolase in vivo, but that it had no significant effect on Fl-enolase. We predicted that, if the actin cytoskeleton was involved in immobilizing the bound fraction of aldolase in our experiments, then disrupting actin organization with CD should increase the mobility of Rh-aldolase, but not Fl-enolase. Our data are consistent with that prediction, and with models in which a fraction of aldolase is bound to the actin cytoskeleton in vivo. The CD-induced reorganization of the actin cytoskeleton may cause the release of aldolase from actin filaments, or may result in small oligomeric actin fragments which remain bound to aldolase. In either case, we would expect to see a decrease in the bound fraction of aldolase using FRAP.

Both 2-DG and CD caused a small, global decrease in the D_{cyto} of both Rh-aldolase and Fl-enolase. Although it is not clear that this decrease is significant, due to the errors inherent in measurements in vivo, it has been a consistent finding in our experiments, and it is possible that it reflects an increase in cytoplasmic apparent viscosity. In the case of 2-DG, release of bound aldolase molecules (and perhaps other glycolytic enzymes) could cause increased cytoplasmic micro-viscosity; in the case of CD, an increase in the number of actin molecules (due to fragmenting of filaments) could contribute to increased cytoplasmic micro-viscosity. Alternatively, if CD action in vivo results in aldolase bound to small oligomeric actin fragments, we would also expect to measure a slightly decreased D_{cyto} value with FRAP.

Aldolase as an Ambiquitous Enzyme

Ambiquitous enzymes were originally defined by Wilson (1978) as having kinetically distinct subsets which partition between soluble and membrane-bound forms in cells. Ambiquity provides a model for regulation of cytoplasmic enzyme activity, using a mechanism analogous to allostery, in which binding of an enzyme to a structural element regulates its catalytic activity. Hexokinase was subsequently identified as the prototype ambiquitous enzyme (Wilson, 1980), and evidence has recently been presented that hexokinase partitions between bound and free fractions in vivo (Laursen et al., 1990) and that 2-DG modulates the association of hexokinase with mitochondria in fixed cells (Lynch et al., 1991). The structural element(s) to which an ambiquitous enzyme

binds are not limited to membrane-associated structures; indeed, relatively weak interactions among several enzymes and cytoskeletal components could be significant at the high concentrations found in cytoplasm (Walsh and Knull, 1987). The binding of aldolase to the actin cytoskeleton could modulate the activity of aldolase; conversely, inhibition of glycolysis with 2-DG could modulate the ratio of bound/free aldolase. The data in this paper are consistent with the last model, and we believe that our evidence suggests that aldolase displays ambiquitous behavior in 3T3 cells. Enolase has no intracellular bound fraction, and shows no evidence of ambiquitous behavior.

Ambiquity may account, in part, for a paradoxical finding in this study and in our previous work (Pagliaro and Taylor, 1988). Initially, we predicted that the high actin concentration in the cell periphery would result in a greater bound fraction of aldolase relative to the perinuclear region. We have consistently observed the opposite; a bound fraction of aldolase in the perinuclear region, and 100% mobility in the cell periphery. In addition, however, we have also consistently measured a substantial (40-50%) lower D_{cyto} in the cell periphery relative to the perinuclear region. It is not clear from our data that the reduced peripheral D_{cyto} is significant, due to the large errors inherent in FRAP measurements in vivo, but transient interactions between aldolase and the actin cytoskeleton, which are faster than the time resolution of our FRAP instrument, could account for the lower peripheral D_{cyto} we measured. Thus, the bound fraction of aldolase in the perinuclear region may represent a kinetically distinct, downregulated subset of aldolase, while the fully mobile (but lower D_{cyto}) peripheral measurements may average brief bound and mobile diffusion components. Such rapid, transient interactions could represent dynamic ambiquitous behavior of aldolase.

Do Glycolytic Enzymes Exhibit Functional Duality?

Functional duality, as defined by Clarke et al. (1985a), describes a more general case than does ambiquity: a glycolytic enzyme could possess significant biological activities in addition to its primary catalytic role. Thus, the bound phase of aldolase may serve other functions typical of actin-binding proteins; indeed, aldolase may behave as a true actin-binding protein under some metabolic conditions (Maciver et al., 1991). It is clear that aldolase is an actin-gelation factor in vitro (Clarke et al., 1985a; Pagliaro and Taylor, 1988), and it could be involved in cytoplasmic integration or tension-transmission in vivo (Luby-Phelps et al., 1988). Functional duality could associate glycolytic metabolism with cytoplasmic structure and cell motility in a broader physiological context than conventional metabolic models allow for.

ATP Metabolism and Cytoskeleton Regulation

There is substantial evidence that ATP metabolism is closely related to the regulation and stability of the actin cytoskeleton (Bershadsky and Gelfand, 1983). A variety of respiratory inhibitors (oligomycin, CCCP, sodium azide and 2,4-DNP) caused a gradual (90 min) disorganization of the actin cytoskeleton in mouse embryo fibroblasts, when the cells were cultured in glucose-free medium (Bershadsky et al., 1980). When glucose was present in the culture medium, however, the actin distribution was not affected, showing that glycolytic ATP alone is sufficient to maintain the integrity

of the actin cytoskeleton. Sanger et al. (1983) have demonstrated that actin-containing bundles had differential sensitivity to cellular ATP levels; stress fibers were more resistant to ATP depletion (using 2-DG together with respiratory inhibitors) than cleavage rings (Sanger et al., 1983). Normal stress fiber patterns were restored after inhibitors were washed out in a subsequent report from the same group (Glascott et al., 1987). Finally, it was reported that primary *Xenopus* tadpole heart cells were more sensitive to metabolic inhibitors than was an established cell line from the same source (Bereiter-Hahn et al., 1984). These studies provide evidence that glycolytic ATP metabolism is essential for modulating the actin cytoskeleton. Further, they suggest that such modulation could be specific for stress fibers, rather than other actin-based structures, and that it may be developmentally regulated.

Although we know far more about the organization of cytoplasm today than we did when the term "cytosol" was operationally defined (Lardy, 1965), our working assumptions about intrinsically cytoplasmic biochemistry (such as glycolytic metabolism) have evolved relatively little. It is clear, for example, that weak interactions can become very important given the high molar concentrations and complex equilibria of cytoplasm. We believe that experiments in living cells will be essential for further defining the organization and regulation of metabolic pathways and all cellular biochemical events.

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