Enolase exists in the fluid phase of cytoplasm in 3T3 cells

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Summary

We have investigated the intracellular distribution and mobility of the glycolytic enzyme enolase, using functional fluorescent analogs labeled with the succinimidyl esters of carboxyfluorescein (Fl-enolase) and carboxytetramethylrhodamine (Rh-enolase). In contrast to aldolase, neither native enolase nor labeled enolase gelled filamentous actin (F-actin), as measured by falling-ball viscometry, indicating a lack of interaction between enolase and F-actin. Fluorescence redistribution after photobleaching (FRAP) measurements of the diffusion coefficient (D) of Fl-enolase in aqueous solutions gave a value of D_{aq} = 6.08 \times 10^{-7} \text{cm}^2\text{s}^{-1}, and no immobile fraction, consistent with a native molecular weight of 90 000. These values were not significantly different with Rh-enolase, or in the presence of F-actin, 2-phosphoglycerate or F-actin-aldolase gels, demonstrating that neither Fl-enolase nor Rh-enolase binds to F-actin or aldolase in vitro. FRAP measurements of Fl- and Rh-enolase microinjected into living Swiss 3T3 cells revealed spatial differences in the diffusion coefficient, but not the mobile fraction. In the perinuclear cytoplasm, we measured an apparent diffusion coefficient of 1.1 \times 10^{-7} \text{cm}^2\text{s}^{-1}, compared to 7.1 \times 10^{-8} \text{cm}^2\text{s}^{-1} in the peripheral cytoplasm, with \approx 100\% mobility of Fl- or Rh-enolase in both regions. Imaging of cells co-injected with Rh-enolase and size-fractionated FITC-dextran (FD-90) revealed that Rh-enolase entered the nucleus, while FD-90 was excluded. Ratio imaging showed a relatively high nuclear ratio of Rh-enolase/FD-90, and a uniform cytoplasmic ratio, with no indication of increased concentration of enolase around stress fibers. These data demonstrate that Rh- and Fl-enolase do not bind to F-actin in vitro, and are 100\% mobile in vivo. Together with our recent finding that a significant fraction of aldolase binds to F-actin in vitro and is immobile in vivo, these data suggest a correlation between actin-binding activity and cytoplasmic mobility of glycolytic enzymes.

Key words: enolase, cytoplasm, diffusion coefficient.

Introduction

In the quarter century since the aqueous compartment of the cytoplasm was termed the 'cytosol' (Lardy, 1965), considerable evidence that cytoplasm is highly organized and structured has accumulated (Welch, 1977; Clegg, 1984a,b; Porter, 1984; Bhargava, 1985; Gershon et al. 1985; Srere, 1987; Srivastava & Bernhard, 1987; Luby-Phelps et al. 1988). In spite of this evidence, however, the paradigm that governs most current thinking and research about the cytoplasm is that of a concentrated aqueous solution in which reactions are diffusion limited. In particular, the reactions of glycolysis are generally presumed to take place in a concentrated aqueous solution in which reactions are diffusion limited. In particular, the reactions of glycolysis are generally presumed to take place in solution (de Duve, 1972; Lehninger, 1973), although there is significant evidence to question this assumption (Masters, 1984; Clarke et al. 1985; Friedrich & Hajdu, 1987). A clear demonstration that glycolysis occurs and/or is regulated partially in association with one or more solid phases of cytoplasm would help define a new level of organization in the cell.

We have recently found that aldolase binds reversibly to F-actin in vitro, has an immobile fraction in living Swiss 3T3 cells, and appears concentrated in a zone around stress fibers (Pagliaro & Taylor, 1988). We predicted that, if the actin-binding activity of aldolase was responsible for the immobile fraction we observed in vivo, then a glycolytic enzyme lacking actin-binding activity in vitro should also lack an immobile fraction in vivo. Enolase (see Table 1) was an ideal candidate for this study, since it has no (Bronstein & Knull, 1981), or very little (Arnold et al. 1971; Clarke & Masters, 1976; Walsh & Knull, 1988), direct actin-binding activity. In addition, enolase is the only glycolytic enzyme that does not demonstrate indirect, or 'piggyback', binding to a filamentous actin (F-actin) affinity column in the presence of a myogen preparation containing all of the glycolytic enzymes (Bronstein & Knull, 1981). Since adult enolases are homodimers (Fletcher et al. 1976; Russell et al. 1986), the opportunity for divalent binding to, or cross-
linking of F-actin would exist if the appropriate binding sites were present.

We have tested the above prediction by developing functional fluorescent analogs of rabbit muscle enolase (fluorescein (Fl)-enolase and rhodamine (Rh)-enolase), and characterizing their activities in vitro and in vivo. We mapped the intracellular distribution and mobility of enolase microinjected into Swiss 3T3 cells using fluorescence digital imaging microscopy. We show here that enolase does not bind to F-actin in vitro, does not have an immobile fraction in vitro, and is not concentrated in a zone around stress fibers.

Materials and methods

Materials

All biochemicals were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise specified.

Fluorescent labeling of enolase

Purified rabbit muscle enolase was reacted with 3-(and-6)-carboxyfluorescein succinimidyl ester or 5-(and-6)carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR) to make fluorescein(Fl)- or rhodamine(Rh)-conjugated enolase, respectively, by a method similar to that used recently for aldolase (Pagliaro & Taylor, 1988). A 2-8 M-ammonium sulfate suspension containing 2 mg of enolase was dialyzed to equilibrium against 1000 volumes of labeling buffer (3 mM-Tris-HCl, 1 mM-MgCl₂, 50 mM-KCl, 1 mM-EDTA, and 0-1 mM-dithiothreitol at pH 7.4). The enolase solution was then dialyzed to 6 mg ml⁻¹ by vacuum dialysis on ice against fresh labeling buffer. The dye was dissolved in dimethyl formamide (DMF; Eastman Kodak Co., Rochester, NY), shortly before use, to make a 10 mg ml⁻¹ stock solution. Immediately before reaction, a tenfold molar excess of the dye solution (based on the dimeric weight of enolase) was dissolved shortly before use, to make a 10 mg ml⁻¹ stock solution.

Immediately before reaction, a tenfold molar excess of the dye solution was added dropwise with continual stirring to the enolase, the dialyzed enolase solution (based on the dimeric weight of enolase) was dissolved and incubated on ice for 20 min. The reaction was terminated by adding sufficient 1 M-ethanolamine to bring the final concentration to 100 mM; the mixture was then stirred on ice for an additional 3 min. The reacted mixture was dialyzed against three changes of labeling buffer in the dark on ice for 3-4 h using collodion bags (Schleicher and Schuell, Inc., Keene, NH) before gel filtration chromatography. The final concentration of Fl- or Rh-enolase was 0.9-1.0 mg ml⁻¹, and it could be stored on ice for one week with an enzymatic activity loss of 1-2% day⁻¹. For assays of activity, parallel batches of enolase were prepared in which a control was treated identically to the Fl- or Rh-enolase, except that no dye was present in the sample of DMF that was added to the reaction buffer (unlabeled controls).

Protein assay

The protein assay used in these experiments was adapted from that of Schacterle & Pollack (1973). All standards and samples were read at 750 nm (instead of 650 nm) in order to minimize interference from the fluorochrome absorbance maxima at 495 nm (for Fl-enolase) and 557 nm (for Rh-enolase) in the labeled species. The dye:protein ratios (D/P) of the labeled species were calculated by standard methods (Simon & Taylor, 1986), using molar extinction coefficients of 75 000 M⁻¹ cm⁻¹ at 495 nm for carboxyfluorescein succinimidyl ester, and 82 000 M⁻¹ cm⁻¹ at 557 nm for carboxytetramethylrhodamine succinimidyl ester (product data from Molecular Probes). The extinction coefficient of current batches of the rhodamine dye is higher than that used in our previous calculations for aldolase labeling (82 000 versus 55 000; Pagliaro & Taylor, 1988). This change reduces the calculated molar D/P (dye:protein ratio), but yields similar fluorescence intensity in protein conjugates.

Fluorescence spectroscopy

Appropriately diluted samples of free dye or labeled enolase in the presence or absence of 2-PG (2-phosphoglycerate) were scanned using the photon counting mode of a spectrophotometer system (Fluorolog 2, SPEX Industries, Inc., Edison, NJ). For fluorescein excitation spectra, emission at 519 nm was monitored, and the excitation wavelength for emission spectra was 495 nm. For rhodamine excitation spectra, emission at 575 nm was monitored, and the excitation wavelength for emission spectra was 557 nm. A slit bandwidth of ~1 nm was used for both excitation and emission measurements. Rayleigh scatter peaks, at 519 nm and 495 nm for fluorescein excitation and emission spectra, and at 575 nm and 557 nm for rhodamine excitation and emission spectra, respectively (3-4 points each), were removed manually after the scans. One cycle of 5 point running average smoothing was used on each spectrum (Savitzky & Golay, 1964).

Column chromatography

A 10 mm×45 cm Sephacryl S-300 (Pharmacia, Inc., Piscata-
Enzymatic activity assay

To assess the catalytic activity of Fl-enolase, an Enzymatic activity assay was performed as described previously (Pagliaro & Taylor, 1988; Clarke et al., 1985; MacLean-Fletcher & Pollard, 1980; Simon et al., 1988). Actin prepared by the method of Spudich & Watt (1971) was used at a final concentration of 1.0 mg/ml, and the assay buffer consisted of 50 mM-KCl, 1 mM-MgCl₂, 0.5 mM-dithiothreitol, and 10 mM-imidazole, at pH 6.8. A 300 µl portion of each sample was prepared and drawn into three 100 µl capillaries (Clay Adams, Parsippany, NJ). The tubes were capped with “modeling clay,” incubated for 30 min in a water bath at 37°C, measured in triplicate and the times averaged. Straight lines were fit to inverse velocities with a microcomputer spreadsheet using slope and intercept values determined with standard glycerol solutions (Fowler & Pollard, 1982).

**FRAP and calculations of diffusion coefficients**

*In vitro* and *in vivo* FRAP experiments were performed by the Gaussian spot method of Axelrod et al. (1976) essentially as described previously (Pagliaro & Taylor, 1988). Briefly, the 514 nm (for rhodamine) and 488 nm (for fluorescein) lines of an ion argon laser (Spectra Physics) were focused through a Universal microscope equipped with epifluorescence optics (Carl Zeiss, Inc., Thornwood, NY). A 16× Plan-achromat objective (Carl Zeiss; NA = 0.35) was used for *in vitro* measurements, and a 63× Plan-Neofluar oil-immersion objective (Carl Zeiss; NA = 1.25) was used for *in vivo* measurements. Cells were microinjected as for imaging experiments (see below). Bleaching times ranged from 5 to 50 ms, and 15–25% bleaching gave the most consistent data. Spot radii, measured as described previously (Luby-Phelps et al., 1985, 1986), were between 4 and 18 µm, and fluorescence recovery was monitored for 12–18 s, which represented 15–30 halftime of recovery. FRAP recovery curves were analyzed by the method of Yguerabide et al. (1982), and percentages of mobile fractions, defined as the % recovery in the experimental timecourse, were calculated according to Axelrod et al. (1976). Data acquisition and analysis were performed using a program written in Asyst (Macmillan Software Co., New York, NY) and procedures used were similar to those used previously (Pagliaro & Taylor, 1988; MacLean-Fletcher & Pollard, 1980). Data analysis and statistical analysis were performed using a program running on a 16 MHz 80386 microcomputer (Dell Computer Corp., Austin, TX).

**Cell culture and preparation**

Passage numbers 121–132 Swiss 3T3 fibroblasts (ATCC-CCL92, American Type Culture Collection, Rockville, MD) were cultured and prepared as described previously (DeBiasio et al. 1987).

### Table 2. In vitro FRAP data

<table>
<thead>
<tr>
<th>Sample</th>
<th>n*</th>
<th>$D_{20,40} \times 10^7$</th>
<th>$D_{37,40} \times 10^7$</th>
<th>% Mobile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fl-enolase (1 µm)</td>
<td>20</td>
<td>3.96 ± 0.28</td>
<td>6.08 ± 0.43</td>
<td>99.1 ± 3.9</td>
</tr>
<tr>
<td>Fl-enolase + 2.5G (100 µm)</td>
<td>15</td>
<td>3.98 ± 0.28</td>
<td>6.09 ± 0.43</td>
<td>103.7 ± 3.5</td>
</tr>
<tr>
<td>Fl-enolase + F-actin (1 mg ml⁻¹)</td>
<td>11</td>
<td>3.99 ± 0.27</td>
<td>6.11 ± 0.41</td>
<td>105.0 ± 0.5</td>
</tr>
<tr>
<td>Fl-enolase + F-actin + aldolase (1 µm)</td>
<td>17</td>
<td>3.90 ± 0.12</td>
<td>5.06 ± 0.18</td>
<td>107.1 ± 1.1</td>
</tr>
<tr>
<td>Rh-enolase</td>
<td>16</td>
<td>3.93 ± 0.21</td>
<td>6.03 ± 0.32</td>
<td>101.0 ± 1.9</td>
</tr>
<tr>
<td>Rh-enolase + F-actin</td>
<td>14</td>
<td>3.80 ± 0.17</td>
<td>5.83 ± 0.27</td>
<td>100.4 ± 2.2</td>
</tr>
<tr>
<td>FD-90 (1 µm)</td>
<td>17</td>
<td>3.79 ± 0.36</td>
<td>5.81 ± 0.55</td>
<td>104.6 ± 2.5</td>
</tr>
<tr>
<td>FD-90 + F-actin</td>
<td>16</td>
<td>3.75 ± 0.20</td>
<td>5.75 ± 0.30</td>
<td>102.7 ± 1.0</td>
</tr>
</tbody>
</table>

* Number of measurements used in calculating the mean.
† Aqueous diffusion coefficients (cm²s⁻¹) were measured at 22°C–24°C and normalized to 20°C ($D_{20,40}$) and to 37°C ($D_{37,40}$) for comparison to *in vivo* data.
‡ Mean ± the sample standard deviation.

Falling ball viscometry and actin

Falling ball viscometry was used to measure the apparent viscosities of mixtures of enolase and F-actin; the conditions and procedures used were similar to those used previously (Pagliaro & Taylor, 1988; Clarke et al., 1985; MacLean-Fletcher & Pollard, 1980). The reaction mixture consisted of 50 mM-imidazole, 1 mM-MgCl₂, 0.5 mM-dithiothreitol, and 10 mM-imidazole, at pH 6.8 (IKMD). A 300 µl portion of each sample was prepared and drawn into three 100 µl capillaries (Clay Adams, Parsippany, NJ). The tubes were capped with ‘modeling clay,’ incubated for 30 min in a water bath at 37°C, measured in triplicate and the times averaged. Straight lines were fit to inverse velocities with a microcomputer spreadsheet using slope and intercept values determined with standard glycerol solutions (Fowler & Pollard, 1982).

**Cell culture and preparation**

Passage numbers 121–132 Swiss 3T3 fibroblasts (ATCC-CCL92, American Type Culture Collection, Rockville, MD) were cultured and prepared as described previously (DeBiasio et al. 1987).
Microinjection and imaging

Approximately 5–10% of a cell volume of a stock solution containing 3–4 mg ml⁻¹ of Rh- or Fl-enolase (equal to 4–8% of the cellular enolase pool) and/or FD-90 was microinjected into 3T3 cells. Injected cells were allowed to recover for 30 min to 2 h, then placed in a modified Sykes-Moore chamber on a temperature-controlled stage and imaged with the system described by Bright et al. (1987). Briefly, a 63× Plan-Neofluar oil-immersion objective (1.25 NA), with narrow-bandpass rhodamine and fluorescein epifluorescence filter sets on a Universal microscope (Carl Zeiss, Inc.), was used to image the fluorescence signal on an ISIT camera (Dage-MTI, Inc., Michigan City, IN). Images of 128 averaged video frames were background subtracted and processed on an image processor (VDP 1800, VICOM Systems, Inc., Junction City, CA), and photographed with HP-5 film (Ilford Limited, Cheshire, England) from the high-resolution display monitor. Rhodamine and fluorescein image pairs (for Rh-enolase and FD-90, respectively) were processed similarly. Pairs of images for ratios were acquired sequentially within 20 s. Ratios were calculated and normalized to a scale of 0–0–1 by a floating-point method (Rogowska et al. 1989).

Results

In order to address fully the issue of fluorophore-induced artifacts, we have done two parallel studies: we duplicated all of our initial findings using Fl-enolase with Rh-enolase, and consistently found that no significant differences existed between the two sets of data. We considered the possibility that the relatively hydrophobic carboxytetramethylrhodamine succinimidyl ester might influence the behavior of Rh-enolase, and cause it to behave differently than Fl-enolase. Such differences might occur in vivo, but not in vitro. For example, the rhodamine dye (but not the fluorescein dye) could partition preferentially into membrane compartments in vivo. The only way to address this important concern unambiguously was to do parallel studies with both fluorophores. With our present results, we feel that mobility comparisons between molecules labeled with the succinimidyl esters of rhodamine and fluorescein are fully justified.

Characterization of enolase labeling

One-dimensional SDS–polyacrylamide gel electrophoresis of Fl- and Rh-enolase samples revealed a single band with a \( M_r \) of approximately 46,000 (data not shown). Labeled and unlabeled molecules migrated identically. Dialysis in collodion bags was effective for separation of the free dye from the labeled protein, but significant amounts of higher- and lower-molecular weight protein contaminants necessitated gel filtration chromatography of the labeled protein. After dialysis and chromatography, very little free dye was visible at or ahead of the dye front of the gel under ultraviolet illumination, and the single 46,000 \( M_r \) band was both fluorescent and Coomassie Blue staining.

We used both Fl-enolase and Rh-enolase with a spectrophotometrically determined molar \( D/P \) of 2:0:1. This labeling yielded one fluorophore/monomer, which provided a good compromise between reduction in activity and fluorescence intensity.

Fig. 1. Excitation and emission spectra of labeled enolase. Spectra for Fl-enolase in the presence (—) and absence (· · · · · ·) of a 100-fold molar excess of 2-PG, and Rh-enolase in the presence (—) and absence (· · · · · ·) of a 100-fold molar excess of 2-PG. The spectra for Rh-enolase were measured from a more dilute solution and multiplied by a factor of 10 (×10) for comparison with the Fl-enolase spectra.

Fl-enolase had excitation and emission maxima of 495 nm and 519 nm, respectively, yielding a Stokes' shift of 24 nm, and Rh-enolase had absorption and emission maxima at 557 nm and 575 nm, respectively, yielding a Stokes' shift of 18 nm (Fig. 1). Spectra taken in the presence of a 100-fold molar excess of 2-PG were nearly identical, showing no significant absorbance changes or spectral shifts upon substrate binding. In addition, the spectra of both Fl-enolase and Rh-enolase closely resemble those of the free fluorescein and rhodamine dyes, respectively, when normalized for fluorescence intensity (data not shown).

Fl-enolase is biologically active

Fl- and Rh-enolase eluted from an S-300 column with a profile similar to native enolase (Fig. 2), demonstrating that the labeled molecule retained dimeric conformation. The 5% lead of the labeled species (\( K_{av} \) of Fl-enolase = 0.381 versus \( K_{av} \) of enolase = 0.404) was similar to that seen with labeled aldolase (Pagliaro & Taylor, 1988), and is probably due to charge effects of the fluorophore.

Both Fl- and Rh-enolase (\( D/P = 2:0:1 \)) retained 85% of the catalytic activity of the native molecule. Fl-enolase had enzymatic activity only 1–2% lower than that of the unlabeled control, demonstrating that the labeling conditions (pH shift, DMF, ethanolamine), and not fluorophore binding directly, were primarily responsible for the 10–15% loss of activity in both groups. 55% of the catalytic activity (\( V_{max} \)) of Fl-enolase remained after laser irradiation equivalent to the bleaching dose used in FRAP experiments (data not shown).

We used falling ball viscometry to assay for actin-gelling activity of enolase and Fl- or Rh-enolase. Enolase at concentrations from 1 to 9 \( \mu \)M did not gel 1 mg ml⁻¹
Enolase does not bind to F-actin in vitro

• $D_{aq} = 6.08 \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$, with no immobile fraction, and this was not significantly changed in the presence of F-actin. It is clear from these data that neither Fl- nor Rh-enolase binds to F-actin in vitro.

Aqueous FRAP measurements of FD-90 fractions with gel filtration $K_g$ values similar to those of enolase, had an average mobility of $D_{aq} = 5.81 \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$, which was not significantly affected by the presence of F-actin.

Enolase has no immobile fraction in vivo

We used FRAP measurements to determine the mobilities of Fl- and Rh-enolase as well as FD-90 in living 3T3 cells (Table 3). Since we found significant spatial differences within individual cells in both the diffusion coefficient and the mobility of aldolase (Pagliaro & Taylor, 1988), we performed FRAP measurements in perinuclear and peripheral regions as shown in Fig. 3B. Peripheral FRAP sites were areas with well-developed stress fibers (20–40 % of beam area) visible by phase-contrast microscopy; sites free of obvious vesicles and non-adjacent to the centrosome were chosen for perinuclear FRAP.

Fl-enolase had a $D_{cyt}$ (apparent cytoplasmic diffusion coefficient) of $1.1 \times 10^{-7} \text{ cm}^2 \text{s}^{-1}$ in the perinuclear region, a value 5.4-fold lower than its aqueous diffusion coefficient. This reduction is probably explained by a combination of the higher viscosity of the aqueous phase of the cytoplasm, which has been reported to be between 3 and 6 cP (Lepock et al. 1983; Mastro et al. 1984; Paine & Horowitz, 1980), and hindered diffusion due to cytoplasmic structure (Luby-Phelps et al. 1987). The diffusion coefficient in the cell periphery was $7.2 \times 10^{-8} \text{ cm}^2 \text{s}^{-1}$, a reduction of about 35 % compared to that measured in the perinuclear region. Significantly, we found no immobile fraction of enolase in either perinuclear or peripheral cytoplasm, demonstrating that enolase is 100 % mobile in 3T3 cytoplasm during the timecourse of our FRAP measurements. In vivo FRAP values for Rh-enolase were very similar to those for Fl-enolase.

We found no immobile fraction of FD-90 in either perinuclear or peripheral regions. The diffusion coefficients of FD-90 were intermediate between the two enolase values, and showed smaller spatial differences than enolase did. The peripheral diffusion coefficient of FD-90 was 90 % of the perinuclear diffusion coefficient, a significant difference from the 35 % reduction in enolase diffusion coefficient seen in the cell periphery.

Enolase enters the nucleus, but is not concentrated around stress fibers

Imaging of cells co-injected with Rh-enolase and FD-90 revealed significant differences between the distributions of enolase and FD-90 (Fig. 3). Enolase injected into the cytoplasm entered the nucleus rapidly (within a 30 min recovery time), yet had a very even cytoplasmic distribution. Exclusion of enolase from nucleoli and vesicles was evident (Fig. 3A). Although FD-90 has a hydrodynamic radius similar to enolase, based on its elution
Fig. 3. Fluorescence images of a cell co-injected with Rh-enolase and FD-90. Rh-enolase (A) entered the nucleus, but was excluded from nucleoli. FD-90 (B) was excluded from the nucleus, but had a cytoplasmic distribution similar to that of Rh-enolase. Circles in B indicate typical perinuclear (large arrowhead) and peripheral (small arrowhead) sites. Bar, 10 μm.

Fig. 4. Ratio image of enolase distribution. The Rh-enolase image of Fig. 3A was divided by the FD-90 image of Fig. 3B.
Fig. 5. A stress fiber-rich cell periphery; Rh-enolase image (A), FD-90 image (B), and ratio image (C). Concentration of Rh-enolase around stress fibers is not evident.

profile, FD-90 clearly does not enter the nucleus, though it has similar exclusion from cytoplasmic vesicles (Fig. 3B). In order to compare the distributions of enolase and FD-90 directly, we generated a ratio image of Rh-enolase fluorescence/FD-90 fluorescence (Fig. 4; Tanasugarn et al. 1984; Bright et al. 1987, 1989; Rogowska et al. 1989). Higher ratio values, indicated by brighter areas in the ratio, indicate a high concentration of enolase relative to FD-90, notably the nucleus in Fig. 4. The cytoplasmic ratio intensity was quite even, indicating similar distributions of enolase and FD-90 in the cytoplasm.

Even in the periphery of a well-spread, stress fiber-rich fibroblast (Fig. 5), there were few differences between the distributions of Rh-enolase and FD-90, as demonstrated by the relatively uniform ratio image (Fig. 5C). Slight exclusion of FD-90 from stress fibers can be seen in Fig. 5B. By cropping the nucleus from this set of images, we maximized the sensitivity of the ratio program for small differences in the cytoplasmic concentrations of Rh-enolase and FD-90 (Rogowska et al. 1989). These conditions revealed neither concentration of enolase in, nor exclusion from, any part of the peripheral cytoplasm. The increased ratio at the edge of the lamellum in Figs 4 and 5C is probably due to cell movements during the interval between successive images. The distribution of Fl-enolase appeared identical to that of Rh-enolase (data not shown).

Discussion

This study establishes that enolase neither binds to F-actin in vitro, nor has an immobile fraction in living Swiss 3T3 cells. Together with our recent finding that aldolase binds to F-actin in vitro, has a ≈23% immobile fraction in the perinuclear region in vivo, has a twofold reduced mobility in the actin-rich cell periphery, and exhibits a local concentration around stress fibers (Pagliaro & Taylor, 1988), these data are consistent with models in which some, but not all, glycolytic enzymes bind to the actin cytoskeleton as a form of metabolic compartmentation (Masters, 1984; Clegg, 1984b). For
aldolase and enolase, we have demonstrated a correlation between actin-binding activity in vitro and an immobile fraction in vivo. Since aldolase, but not enolase, is concentrated in a zone around stress fibers, all glycolytic enzymes do not appear to be compartmented equally. Actin-binding activity could play a role in the compartmentation and/or regulation of some glycolytic enzymes, but not others.

The exclusion of FD-90 from nuclei is consistent with previous data on diffusion of dextrans into nuclei; the nuclear exclusion limit appears to be about 70 000 Mr, for labeled dextrans (Jiang & Schindler, 1986, 1988; Paine et al. 1975). Consequently, our observation that enolase enters the nucleus is surprising. Enolase may be actively transported through nuclear pores that exclude dextrans (and some proteins) of similar hydrodynamic radius (Feldherr et al. 1983; Jiang & Schindler, 1986; Schindler & Jiang, 1986). Alternatively, enolase may dissociate into monomers that can pass through nuclear pores after microinjection, although this is unlikely on the basis of the D cyt0 values we have measured. In contrast to enolase, both rhodamine–aldolase and similarly size-fractionated 150 000 Mr, FITC–dextran are excluded from the nucleus of Swiss 3T3 cells (Pagliaro & Taylor, 1988).

Fluid versus solid phases of cytoplasm
We have operationally defined cellular components as part of either the fluid or solid phase of cytoplasm on the basis of the absence or presence of an immobile fraction detected with the time resolution of our FRAP measurements. We believe we have detected significant, biologically relevant differences in the intracellular behavior of enolase and aldolase (Pagliaro & Taylor, 1988) using fluorescent analog cytochemistry and FRAP. However, we refer to 'fluid' and 'solid' phases of cytoplasm cautiously, and in a relative sense. The evolving picture of cytoplasmic organization is quite different from the traditional view in which reactions in the non-membrane-bound 'cytosol' (Lardy, 1965) are largely diffusion limited (Ottaway & Mowbray, 1977; Clegg, 1984a; Masters, 1984). Since it is well established that some glycolytic enzymes bind to actin filaments in vitro, it is possible that at any instant there is a fraction of enzyme bound to structural components in vivo. This is reflected differently in a FRAP record depending on the on–off rate of the enzyme relative to its D cyt0 and the pattern size generated by the FRAP instrument.

What we observed as an immobile fraction of enzyme in the case of aldolase (Pagliaro & Taylor, 1988) may represent a catalytically distinct subset of the cellular pool of that enzyme (Arnold & Pette, 1970; Walsh et al. 1977). The absence of an immobile fraction of enolase in vivo does not suggest 100 % catalytic activity, since enolase lacks actin-binding activity. Rather, it suggests that enolase may be regulated differently from actin-binding glycolytic enzymes, such as aldolase.

**Enolase as a cytoplasmic marker**
Studies of protein mobility in vivo have traditionally lacked an ideal control molecule. Early attempts to use exogenous proteins such as bovine serum albumin and ovalbumin as diffusible controls had limited success due to significant immobile fractions of those proteins, attributed to non-specific binding (Taylor & Wang, 1978; Wang et al. 1982; Luby-Phelps et al. 1985). More recently, studies in our laboratory and others have used neutral dextrans and Ficolls as diffusible volume indicators in living cells (Luby-Phelps et al. 1985, 1986; Schindler & Jiang, 1986; Paine & Horowitz, 1980).

While size-fractionated neutral dextrans and Ficolls do not exhibit immobile fractions in vivo, they have moderately polydisperse distributions of molecular weights ranging around a mean value (Bohrer et al. 1984).

The ideal control molecule for in vivo mobility measurements would be a non-binding, spherical, monodisperse particle. Enolase could potentially be the first protein to fill this role, but several issues must be addressed first. Enolase exhibits neither actin-binding activity in vitro nor an immobile fraction in vivo. However, the peripheral D cyt0 value of Rh- and Fluorochrome–enolase is 35 % lower than the perinuclear D cyt0 value. While this reduction is less than the 50 % decrease in the Rh-aldolase D cyt0 value in the cell periphery (Pagliaro & Taylor, 1988), it is significant. In light of this new evidence, we believe that two components may contribute to the peripheral reduction in D cyt0 of the glycolytic enzymes we have studied. Rapid, transient interactions between diffusing proteins and cellular structures (Gershon et al. 1985) could represent the first component, and hindered diffusion, resulting from a smaller mesh size in the peripheral cytomatrix (Luby-Phelps et al. 1988), could represent the second component. Enolase may be affected only by hindered diffusion at the cell periphery, while aldolase is affected by both transient interactions and hindered diffusion. Although it is possible that a fraction of the enolase pool dissociates into monomer, which is responsible for the higher perinuclear diffusion coefficient, this is unlikely, since single-component recovery curves fit our FRAP data very well ($\chi^2 = 1 \times 10^{-4}$ to $5 \times 10^{-4}$).

The smaller reduction in D cyt0 of FD-90 at the cell periphery (10 %) is probably explained by the relatively elongate structure of dextrans. Dextrans of a given molecular weight are affected less by obstructions to diffusion than are spherical molecules of a similar molecular weight (Bohrer et al. 1984).

**Enolase mobility in axons**
There is about 80 % sequence homology between mammalian muscle-specific (ββ) and nerve-specific (γγ) enolases (McAleese et al. 1988; Ohshima et al. 1989); accordingly, we think it is reasonable to compare work on nerve-specific enolase mobility with our present data. Brady & Lasek (1981) reported that nerve-specific enolase comigrated with neuronal slow component b at 2 mm day$^{-1}$ in guinea-pig optic axons, but that it did not 'appear to have significant affinity for actin'. Thus, their evidence for association of enolase with the axoplasmic matrix does not conflict with our present finding of 100 % enolase mobility, since more than 100 other polypeptides are associated with guinea-pig slow component b. Nerve-specific enolase could associate with the axoplasmic
matrix indirectly, the slow component b polypeptides not present in 3T3 cytoplasm. Alternatively, nerve-specific enolase could have different actin-binding properties and intracellular mobility from the muscle-specific enolase that we have studied, though this is unlikely, given the high homology between enolase isozymes.

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References


