Flight Muscle Function in *Drosophila* Requires Colocalization of Glycolytic Enzymes

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Submitted February 18, 1997; Accepted June 2, 1997
Monitoring Editor: James A. Spudich

Structural relationships between the myofibrillar contractile apparatus and the enzymes that generate ATP for muscle contraction are not well understood. We explored whether glycolytic enzymes are localized in *Drosophila* flight muscle and whether localization is required for function. We find that glycerol-3-phosphate dehydrogenase (GPDH) is localized at Z-discs and M-lines. The glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are also localized along the sarcomere with a periodic pattern that is indistinguishable from that of GPDH localization. Furthermore, localization of aldolase and GAPDH requires simultaneous localization of GPDH, because aldolase and GAPDH are not localized along the sarcomere in muscles of strains that carry *Gpdh* null alleles. In an attempt to understand the process of glycolytic enzyme colocalization, we have explored in more detail the mechanism of GPDH localization. In flight muscle, there is only one GPDH isoform, GPDH-1, which is distinguished from isoforms found in other tissues by having three C-terminal amino acids: glutamine, asparagine, and leucine. Transgenic flies that can produce only GPDH-1 display enzyme colocalization similar to wild-type flies. However, transgenic flies that synthesize only GPDH-3, lacking the C-terminal tripeptide, do not show the periodic banding pattern of localization at Z-discs and M-lines for GPDH. In addition, neither GAPDH nor aldolase colocalize at Z-discs and M-lines in the sarcomeres of muscles from GPDH-3 transgenic flies. Failure of the glycolytic enzymes to colocalize in the sarcomere results in the inability to fly, even though the full complement of active glycolytic enzymes is present in flight muscles. Therefore, the presence of active enzymes in the cell is not sufficient for muscle function; colocalization of the enzymes is required. These results indicate that the mechanisms by which ATP is supplied to the myosin ATPase, for muscle contraction, requires a highly organized cellular system.

INTRODUCTION

The nature and functional significance of interactions between the contractile proteins of muscle cells and the enzymes of the pathways that generate ATP, used by myosin ATPase to enable muscle contraction, have not been extensively addressed in insect flight mus-

cles. We have set out to ascertain whether the enzymes involved in ATP production are specifically localized or organized in flight muscle cells and, if so, whether a specific pattern of localization is required for function. *Drosophila* has proven to be a useful organism for studying the structure and function of components of the contractile apparatus of flight muscles. Many genes with products that are required for flight and have a selectable mutant phenotype, "flightlessness," are often not essential at earlier stages of the life cycle. This has enabled the isolation of mutant alleles of genes that encode contractile proteins and has pro-

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vided a productive strategy for the subsequent analysis of their function and interactions. This subject has been reviewed by Bernstein *et al.* (1993) and more recent studies on the molecular biology of contractile proteins have been reported (Barbas *et al.*, 1993; Cripps *et al.*, 1994; Kronert *et al.*, 1994, 1995; Vigoreaux, 1994).

It is clear that a substantial fraction of the ATP for flight muscle contraction is provided through the glycolytic pathway. Genes from *Drosophila* that encode enzymes of the glycolytic pathway have been well characterized at the molecular level. However, with the exception of *Gpdh*, which encodes glycerol-3-phosphate dehydrogenase (GPDH) (Bewley, 1983; MacIntyre and Davis, 1987), their function has not been intensively studied using genetic approaches. Because of its genetic accessibility, the *Gpdh* gene-enzyme system is an excellent model for beginning an analysis of the functional and structural relationships required for energy production in flight muscle.

GPDH in *Drosophila* is encoded by a single gene that is located at cytogenetic position 26A2-5 (MacIntyre and Davis, 1987). Mutations in *Gpdh* can result in flightlessness (O'Brien and Shimada, 1974; Kotarski *et al.*, 1983), thereby providing a useful selectable phenotype. *Gpdh* can encode three protein isoforms, each produced through differential splicing of the transcript from the single *Gpdh* gene. The isoforms differ from one another only at their C termini (Cook, *et al.*, 1988; von Kalm *et al.*, 1989). All isoforms contain the products of exons 1–6. GPDH-2 has 10 additional amino acids at the C terminus, encoded by exon 7. GPDH-1 does not include the amino acids encoded by exon 7 but has three additional amino acids, Q-N-L, at the C terminus encoded by exon 8. It has been known for some time that isoform production is developmentally regulated. GPDH-1 is the only isoform found in thoracic muscle, and thoracic muscle is the only tissue known to contain this isoform (Bewley and Miller, 1979). We have confirmed this observation at the transcript level using RNase protection and tissue *in situ* hybridization (von Kalm and Pollack, unpublished data).

It is not entirely clear why *Gpdh* mutations result in flightlessness. ATP production from carbohydrate catabolism through the glycolytic pathway is able to proceed in such mutants, at least until NAD⁺ becomes limiting. In addition, NAD⁺ can be generated through a number of other reactions, and ATP might also be provided through other pathways. This suggests that GPDH might be a component of a functional complex of glycolytic enzymes, which must be complete to support energy production for flight. The functional significance of such a complex might be to increase the efficiency of ATP production or alternatively might play a role in a necessary compartmentalization of a metabolite pool. Several lines of indirect evidence and a direct study on GPDH turnover (Sullivan, *et al.*,

1983) indicate that GPDH-1 in thoracic muscle is extremely stable relative to GPDH isoforms present in other cell types. This could be consistent with its participation in an organized structure.

MATERIALS AND METHODS

Drosophila Strains

Wild-type flies used in these studies were Oregon-R. *Gpdh* null alleles have been described previously (Kotarski *et al.*, 1983) and are referred to here as JH231, a P-element insertion in *Gpdh* on the balancer chromosome; and In(2LR)SM-1, Df(2L)cl-7, a second chromosome deficiency, which includes *Gpdh* and *nsp10*, an ethylmethane sulfonate (EMS)-induced cross-reacting material (CRM)[−] GPDH null allele.

Transformation

Constructs for germ line transformations all contained the genomic sequence of *Gpdh* extending from a point ~2500 nucleotides 5' to the transcription start site to a *Pst*-1 site in exon 5. The 3' ends came from the appropriate genomic or cDNA clones to encode a single isoform or a pair of isoforms. These constructions have been described in detail (Wojtas 1996). P-element transformation was conducted using a standard protocol (Rubin and Spradling 1982) and the vector pw8 (Klemenz, *et al.*, 1987). Transformants, w⁺ flies, were characterized for GPDH content using Western blots and histochemical staining of dissected tissues (Skuse and Sullivan, 1985; von Kalm *et al.*, 1989). The levels GPDH and pattern of GPDH expression in each of these are comparable with wild-type. However, the tissue specificity for isoform production is based on differential transcript splicing; therefore in transformant lines, plasmid construction determined the appearance of an isoform in a specific tissue (Wojtas, 1996). For example, in lines carrying constructs able to encode only GPDH-1, this isoform is found in larval tissues that in wild-type flies only contain GPDH-2 and GPDH-3. Similarly in muscle, GPDH-3 is produced in transformant lines only capable of producing GPDH-3, whereas in wild-type flies muscle only contains GPDH-1.

Antibodies

Antibodies against GPDH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been characterized previously, and their specificities have been demonstrated (Skuse and Sullivan, 1985; Sullivan *et al.*, 1985). They both are polyclonal antibodies, raised in rabbits, using enzymes purified by standard biochemical procedures as antigens. Western blots using each antibody have been described previously (Skuse and Sullivan, 1985; Sullivan *et al.*, 1985). Antibodies against α -actinin are mouse monoclonal antibodies provided by Dr. Judith Saide (Boston University, Boston, MA). Antibodies against aldolase are rabbit polyclonal antibodies prepared against denatured aldolase and were obtained from Dr. Dean Tolan (Boston University).

Rhodamine-conjugated, affinity-purified anti-rabbit antibodies raised in goat were purchased from Accurate Chemical and Scientific (Westbury, NY). Fluorescein-conjugated anti-rabbit IgG and IgM antibodies raised in goat were purchased from Chemicon International (Temecula, CA).

Microscopy and Immunolocalization

Processing and immunocytochemical procedures used on plastic-embedded specimens have been described in detail previously (Holtham and Slepecky, 1995) and are summarized here. Flies were dropped into fixative containing 3% freshly made paraformaldehyde, 0.1% glutaraldehyde, and 0.1 M sodium phosphate buffer (PB), pH 7.5, at which time the thorax was separated from the head

and abdomen. After fixation from between 2 and 12 h at room temperature, specimens were washed in sodium phosphate buffer, dehydrated through increasing concentrations of alcohol and propylene oxide, infiltrated in Araldite Durcupan ACM (Fluka, Switzerland), oriented, and hardened at 45°C for 1 wk. One-micrometer sections were cut, placed in a drop of water on subbed slides, and dried down at room temperature. Some sections were stained with methylene blue and Azure II and were studied first by light microscopy to confirm orientation and location of fibers, Z-lines, and M-lines. For immunocytochemistry, Araldite was removed in a solution of propylene oxide, absolute ethanol, and sodium hydroxide. Sections were hydrated from ethanol to water, washed in phosphate buffered saline (PBS), and processed for immunocytochemistry in a humid chamber. Specimens were treated sequentially with goat serum (to block nonspecific activity), primary antibodies diluted 1:1000–1:5000 in PBS, PBS wash, goat serum blocker, secondary antibodies diluted 1:200 in PBS, and PBS wash. Slides were dipped in distilled water to wash off the salt, air dried, and stored in the dark at room temperature until viewed. Before use, they were coverslipped with glycerol:*n*-propyl gallate as mounting medium. They were photographed with Kodak (Rochester, NY) TMax 400 film, using a Zeiss (Oberkochen, Germany) Axioskop, 100× oil immersion lens, and 100-W UV light source.

Preparations and Assay of Myofibrils

Flyer were dissected on ice in cold relaxing buffer (Lang *et al.*, 1980). Thoraces were placed into a tube containing the same fixative used for analysis of sectioned thoraces. Thoraces were gently homogenized and allowed to stand on ice for 30 min. Myofibrils were then centrifuged and washed twice in relaxing buffer, incubated in 10% goat serum, washed again, and incubated for 1 h in a mixture of 1:2000 diluted rabbit anti-GPDH serum and 1:100 diluted monoclonal mouse anti- α -actinin (Saide *et al.*, 1989). The fibers were washed and then reacted for 1 h in a mixture of rhodamine-labeled goat anti-rabbit serum and fluorescein-labeled goat anti-mouse serum, each at a final dilution of 1:200. The fibers were washed and resuspended in 50% glycerol and observed using a 100× oil immersion lens, using the appropriate light source.

Enzyme Assays

Preparation of extracts for GPDH assays and GPDH assays were conducted as previously described (Collier, *et al.*, 1976). Aldolase assays were conducted by spectrophotometrically following the fructose-1,6-biphosphate-dependent oxidation of NADH by measuring absorbance changes at 340 nm with coupling to triose phosphate isomerase (TPI)-GPDH using the enzyme reagents supplied by Sigma (St. Louis, MO).

Flight Assays

Flight assays were performed using flies that carried a single allele of a *Gpdh*-carrying transposon in flies having second chromosome homozygous for *Gpdh* null alleles. Second chromosome insertions were tested as heterozygotes with *Df(2LR)cl-7*. Third and X chromosome insertions were tested as heterozygotes with a wild-type homologue in flies having the *Df(2RL)cl-7/nsp10* genotype for the second chromosome. The ability to fly was determined by placing flies of the appropriate genotype in an empty vial. This vial and a second vial containing food were placed 10 cm apart in a shallow tray filled with water. The tray containing the vials was placed in a large Plexiglas enclosure. Flies were observed for 4 h, and then flyers and nonflyers were counted. Flies still in the original vial or in the water surrounding the vials were considered "nonflyers." Flies in the food vial or on the sides of the enclosure were considered "flyers." All flies from the starting sample were accounted for in each assay.

RESULTS

The relative stability of GPDH-1 in thoracic muscle can be appreciated by comparing *Gpdh*-1 transcript and protein levels during the adult life span (Figure 1). Northern blots (Figure 1A) show that *Gpdh*-2 and *Gpdh*-3 transcripts are found throughout development, but *Gpdh*-1 transcripts first appear during the latter half of the pupal stage, the time of flight muscle growth and differentiation. *Gpdh*-1 transcripts decline sharply in abundance and are not detectable later than 4 d after emergence. This is in striking contrast to the levels of GPDH-1 found in thoracic muscle. Western blots (Figure 1B) of extracts of muscles dissected from adult thoraces at different ages show that levels of GPDH-1 do not change appreciably throughout adult life. Because mRNA for this protein is no longer present, the enzyme must be very stable. One explanation for this high degree of protein stability is that the enzyme is incorporated into a stable structural component of muscle cells.

To determine whether GPDH is specifically localized in muscle, we used indirect immunofluorescent microscopy to examine thoracic sections from 2-d-old wild-type flies in the region of flight muscles. These

Figure 1. Developmental profile of *Gpdh* transcripts and isoforms. (A) Developmental Northern blot. Total RNA from animals collected at the indicated day of development after oviposition was electrophoresed and blotted. Identical blots were probed with DNA fragments from the 3' untranslated regions for each isoform specific transcript. Probes for the transcript of the ribosomal protein *rp49* were used as a control. (B) Developmental Western blot; Extracts were prepared from flies at the indicated number of days after emergence, electrophoresed on 12.5% SDS-polyacrylamide gels, electroblotted, and reacted with anti-GPDH serum as described previously (Skuse and Sullivan, 1985).

Figure 2. Immunocytochemical localization of GPDH in *Drosophila* flight muscle. Incubation of sections of thoraces with primary antibodies indicated was followed by incubation in goat anti-rabbit IgG coupled to fluorescein. (A) Thoracic flight muscle from wild-type flies incubated with anti-GPDH antibodies. (B) Thoracic flight muscle from wild-type flies incubated with preimmune serum. (C) Thoracic flight muscle from the GPDH null strain JH231/Df(2L)cl-7 incubated with anti-GPDH antibodies

were stained for GPDH, and we observed that the great majority, if not all, of GPDH-1 in flight muscles is found localized along the sarcomere of the myofibrils in an ordered pattern of alternating strongly fluorescent and somewhat more weakly fluorescent zones (Figure 2A). Two controls establish the specificity of the immunofluorescent microscopic detection of GPDH. Flight muscle from a strain that is known to carry CRM⁻ GPDH null mutant alleles (Kotarski *et al.*, 1983) shows no localized fluorescence after reaction with the same immune serum (Figure 2B). Wild-type muscle reacted with preimmune serum, because the primary antibody in the indirect immunofluorescence assay also shows no localized fluorescence (Figure

2C). We interpreted the sarcomeric fluorescent pattern as showing fluorescence at Z-discs and at M-lines. This difference in fluorescent intensity of these structures may result from variable distributions of GPDH in Z-discs and M-lines or from the variable amount of these structures that is evident in 1- μ m-thick longitudinal sections of these muscles.

We verified that the site of localization of GPDH fluorescence along the sarcomere coincides with Z-discs and M-lines by using isolated myofibrillar preparations simultaneously reacted with anti-GPDH and anti- α -actinin. α -Actinin is a known Z-disc protein (Saide *et al.*, 1989). Micrographs showing myofibrils obtained from wild-type and GPDH null flies reacted with anti-GPDH and anti- α -actinin and photographed first using Nomarski optics and then fluorescent microscopy are shown in Figure 3. Each sarcomere is delineated by the more dense Z-discs, as seen using Nomarski optics (Figure 3A). Their position coincides with the position of α -actinin localization (Figure 3B). GPDH is localized along the sarcomeres of the isolated myofibrils in a pattern quite similar to that seen in the sectioned material. One band of fluorescence is coincident with the position of α -actinin at Z-discs, and another band is equidistant between the Z-discs, at the position of M-lines (Figure 3C). Nomarski images of myofibrils from the GPDH null strain (Figure 3D) appear identical to those from wild-type flies and show a similar α -actinin staining pattern (Figure 3E). However, they show no pattern of GPDH localization (Figure 3F). Given the above considerations, we will refer to the pattern of sarcomeric GPDH localization as Z-disc and M-line localization.

Sarcomeric structures of flight muscle myofibrils from wild-type and GPDH null flies are indistinguishable using light microscopy (Figure 3, A and D). When viewed at the ultrastructural level, sections showing flight muscles from wild-type flies (Figure 4A) and from GPDH null flies (Figure 4B) are also indistinguishable. Therefore, failure of GPDH and other glycolytic enzymes (see below) to be properly localized is not attributable to gross structural distortion of the sarcomeric organization in *Gpdh* mutant strains but must be the result of the presence or properties of GPDH. It should be noted that we have examined muscle from flies during the first 5 d after emergence. In much older flies, GPDH null mutants have been reported to show premature muscle degeneration (O'Brien and Shimada, 1974).

The demonstration that one glycolytic enzyme, GPDH, is distributed in a specific pattern along the sarcomere raises the question of whether this pattern of localization is related to a function specific to GPDH or whether this pattern reflects a property common to other members of the set of glycolytic enzymes. Accordingly, we have used immunofluorescent microscopy to examine flight muscle sections reacted with

Figure 3. Sarcomeric localization of GPDH in isolated myofibrils. (A) Myofibril from wild-type thoracic muscles viewed using Nomarski optics. Large arrows point to Z-lines. (B) Same myofibril as in A reacted with mouse anti- α -actinin, followed by anti-mouse IgG coupled to fluorescein and viewed using fluorescence microscopy. Large arrows indicate positions of Z-discs. (C) Myofibril as in (A) reacted with rabbit anti GPDH, followed by anti-rabbit IgG coupled to rhodamine. Large arrows indicate Z-discs, and small arrows are positioned at both Z-discs and M-lines (D–F) Myofibrils from *Gpdh* null strain treated and microscopically visualized as in A–C, respectively.

antibodies to the glycolytic enzymes GAPDH and aldolase. In flight muscle from wild-type flies, GAPDH (Figure 5A) and aldolase (Figure 5C) are each localized in the sarcomere in a Z-disc and M-line pattern similar to the pattern of GPDH localization (Figure 2A). Intense labeling for GAPDH or aldolase is absent from other regions of the cytoplasm. Strikingly, in flight muscles of flies carrying *Gpdh* null alleles neither GAPDH (Figure 5B) nor aldolase (Figure 5D) is localized at Z-discs and M-lines of the sarcomere. In the GPDH null strain, GAPDH and aldolase are not localized along the myofibrils, seen as thin dark strips that extend lengthwise along the muscle cell. However, in the *Gpdh* null mutant, GAPDH and aldolase fluorescent labeling are present in the cytoplasmic regions that are between the myofibrils and between the darker circular-oval bodies that are alongside of, but outside of, the myofibrils. These dark bodies are mitochondria, which are tightly packed, adjacent to the myofibrils. These cytoplasmic areas are not easily visualized in the wild-type muscles (Figure 5, A and C), because they are not fluorescent; i.e., they contain no GAPDH or aldolase.

Because GPDH can be manipulated genetically, we began our analysis of the molecular basis of glycolytic enzyme colocalization by determining mechanisms by

which GPDH is localized. GPDH-1 is the only GPDH isoform produced in flight muscle, and GPDH-1 is not present in other tissues. GPDH-1 differs from GPDH-3 only in the tripeptide Q-N-L at its C terminus, raising the possibility that this C-terminal tripeptide plays a role in GPDH-1 localization. To test this hypothesis, we made plasmid constructs that could encode GPDH-1, GPDH-3, or both GPDH-1 and GPDH-3. These were introduced into *Drosophila* using P-element-mediated transformation and resulted in flies that could encode: only GPDH-1, *w*; JH231/*cl-7*(*pw*⁺*Gpdc1*); +/+; only GPDH-3, *w*; JH231/*cl-7*; *pw*⁺*Gpdc3*/*pw*⁺*Gpdc3*; or both GPDH-1 and GPDH-3, *pw*⁺*Gpdg1,3*/*pw*⁺*Gpdg1,3*; JH231/*cl-7*; +/+. The presence of GPDH in muscles of these *Gpdh* transgenic flies has been confirmed by Western blot analysis of extracts of dissected muscles (Figure 6).

Flight muscles of transgenic flies were examined by indirect immunofluorescence microscopy (Figure 7). In flight muscles of flies that produce GPDH-1 (Figure 7A), GPDH is localized along the sarcomere in a pattern identical to that seen in muscles of wild-type flies. But flight muscles from transgenic flies capable of producing only GPDH-3 (Figure 7B) show no Z-disc and M-line staining. However, fluorescence is visible on approximately spherical bodies that lie between the

Figure 4. Ultrastructure of flight muscles from wild-type and *Gpdh* null mutant flies. Longitudinal sections and cross-sections of the myofibrils of wild-type (top panels) and *Gpdh* null strains (bottom panels) are shown.

myofibrils. Simultaneous observation with phase and fluorescence microscopy shows that these bodies are mitochondria. Mitochondria, with similar spacing and of similar numbers, are clearly visible between myofibrils in electron micrographs (Figure 4). In addition, unstained shadows of these mitochondria, located between myofibrils, can be seen in Figure 5, B and D. We have also examined the localization of GAPDH in flight muscles from the *Gpdh* transgenic lines using indirect immunofluorescence microscopy. In flies able to produce GPDH-1, GAPDH is localized at Z-discs and M-lines (Figure 8A) in a pattern indistinguishable from that in wild-type flies. However, in flight muscle of flies that produce only GPDH-3, GAPDH (Figure 8B) is not properly localized but is found in nonmyofibrillar cytoplasmic spaces in a pattern similar to that observed for GPDH (Figure 7B) localization in this strain. Therefore, as in the GPDH null mutants, the analysis of GPDH-3-containing transgenic animals demonstrates that specific localization of GPDH is required for glycolytic enzyme colocalization in the sarcomere.

However, the cellular distribution of GPDH (Figure 2C) and GAPDH (Figure 5B) in GPDH null flies is different from the GPDH (Figure 7B) and GAPDH (Figure 8B) cellular distribution in *Gpdh*-3 transgenic flies. In *Gpdh*-3 transgenics, GAPDH and GPDH co-localize to the mitochondria and not at the Z-discs and M-lines. This suggests that GAPDH may associate with GPDH wherever the GPDH localizes. GPDH-3 is normally found in nonmuscle cells, but its localization in nonmuscle cells has not yet been characterized. However, because GPDH-3 is known to participate with glycerol-3-phosphate oxidase, a known mitochondrial enzyme, in accomplishing the glycerol-phosphate shuttle (MacIntyre and Davis, 1987), an association of GPDH-3 with mitochondria in nonmuscle cells or when expressed in flight muscle cells of transgenic flies is plausible.

A key issue is to determine whether functional consequences result when the three glycolytic enzymes studied here are not properly colocalized. To investigate this issue, we determined whether flies that fail to localize glycolytic enzymes at Z-discs and M-lines

Figure 5. Sarcomeric localization of the glycolytic enzymes aldolase and GAPDH in flight muscle. (A) Flight muscle of wild-type flies reacted with anti GAPDH. (B) Flight muscle of *Gpdh* null flies reacted with anti GAPDH. (C) Flight muscle of wild-type flies reacted with anti-aldolase. (D) Flight muscle of *Gpdh* null flies reacted with anti-aldolase.

have flight muscle activity sufficient to support sustained flight. We compared the flying ability of wild-type flies, *Gpdh* null flies, and transgenic flies carrying constructs that can encode GPDH-1, GPDH-3, or both GPDH-1 and GPDH-3, each in a *Gpdh* null background. Results of these assays are presented in Table 1. Values shown are the number of flies able to reach a food-containing vial, determined using the assay described in Materials and Methods. Flies from the *Gpdh* null strain do not fly, as expected. Flies from all strains capable of producing GPDH-1 are able to fly. Clearly, in the absence of localized GPDH-1, flight ability of *Drosophila* is significantly impaired. The most significant results are from the transgenic flies that produce only GPDH-3. These do not fly any more proficiently than GPDH null mutants, even though GPDH and other glycolytic enzymes are present and active in flight muscle cells from the thoraces of these flies. The few flies from the *Gpdh* null strains and GPDH-3 transgenic strains that are classified as flyers in our assay probably get to the food vial by walking, using the surface tension of the water. We have occasionally observed a fly standing or walking on the surface of the water in our assay chamber. The flight assay we have used is reasonably demanding with respect to its flight requirement. Examination of culture vials containing the flightless strains and observations of these flies on an open surface reveal that these flies can elevate from the surface slightly and

traverse trajectories of a few centimeters. They attempt these abortive flights a few times and then rest. These flightless flies appear to walk normally, but we have not attempted to quantify walking ability.

We have measured the specific activities of GPDH and aldolase in thoraces of flies from wild-type *Gpdh* nulls and each of the transgenic strains to confirm the immunocytochemical results showing that glycolytic enzymes are present in muscles that fail to show the Z-disc and M-line pattern of localization. The activity of GPDH in extracts of thoraces from wild-type flies and transgenic flies (Table 2), al-

Figure 6. Western blots of dissected thoracic muscle. Muscles were dissected and extracts prepared from wild-type and the indicated transgenic flies 1 d after their emergence. Extracts were prepared in SDS sample buffer, electrophoresed, and electroblotted, and the blot was probed as described previously (Skuse and Sullivan, 1985).

Figure 7. Sarcomeric localization of GPDH in flight muscles of transgenic flies. (A) Flight muscle of flies from the line *pgpdh-c1* (producing only GPDH-1) incubated with anti-GPDH antibodies. (B) Flight muscle of flies of the line *pgpdh-c3* (producing only GPDH-3) incubated with anti-GPDH antibodies.

Figure 8. Sarcomeric localization of GAPDH in flight muscles of transgenic flies. (A) Flight muscle of flies of the line *pgpdh-c1* (producing only GPDH-1) incubated with anti-GAPDH antibodies. (B) Flight muscle of flies of the line *pgpdh-c3* (producing only GPDH-3) incubated with anti-GAPDH antibodies. (This section is one adjacent to the section shown in Figure 7B.)

though variable, is within the range that might be expected for a set of lines carrying independent transposons. Aldolase activities in these strains are also similar. Variation of aldolase activity in these strains is evident, but enzymatic activity variation is not correlated with the presence or absence of colocalization. Therefore, in flies that are unable to localize their glycolytic enzymes properly, the *Gpdh* nulls or transgenic strains that encode only GPDH-3, the expected enzyme activities are present in the flight muscles. Quantitative GAPDH assays in extracts containing substantial amounts of GPDH and triose phosphate isomerase, as is the case for flight muscles, are not feasible.

In summary, we have shown that three glycolytic enzymes, GPDH, aldolase and GAPDH, are colocalized at Z-discs and M-lines in sarcomeres of flight muscles of *Drosophila*. GPDH localization is dependent, at least in part, on its C-terminal tripeptide found specifically in the muscle isoform GPDH-1. Furthermore, these results allow the distinction of two aspects of glycolytic enzyme function in muscle, localization and catalytic activity. The presence of catalytically active enzyme is not sufficient for the muscle activity required for normal flight, but rather, glycolytic enzymes must be properly colocalized to enable flight.

DISCUSSION

Our results demonstrate that at least a substantial fraction of three glycolytic enzymes of flight muscle cells are found colocalized at Z-discs and M-lines. We cannot be certain that the total cellular content of these enzymes is localized at Z-discs and M-lines, because a fraction of these enzymes might be contained in myofibrillar compartments, e.g., embedded in the region of the thick filaments, which are not readily available to the antibodies. We have found that glycolytic enzymes readily dissociate from isolated myofibrils during their isolation. We have examined progressive washes of unfixed myofibrils for GPDH content using Western blots and found

Table 1. Flying ability of *Gpdh* null and transgenic flies^a

	Wild-type	<i>Gpdh</i> null	<i>pGpdhc1</i>	<i>pGpdhg1,3</i>	<i>pGpdhc3</i>
Flyers	90	3	77	104	4
Nonflyers	3	49	1	8	97
Total	93	52	78	112	101

^aNumbers are the numbers of flies counted. Flyers are those in the vial containing food and those on the sides of the large container. Nonflyers are the numbers of flies remaining in the original vial and in the surrounding water. Totals are the numbers originally put in the initial vial.

Table 2. Aldolase and GPDH activities in thoraces of wild-type, null, and *Gpdh* transgenic lines

	Wild-type	<i>Gpdh</i> null	<i>pGpdhc1</i>	<i>pGpdhg1,3</i>	<i>pGpdhc3</i>
GPDH ^a	0.124	0.00	0.073	0.069	0.062
Aldolase ^b	9.5	6.7	8.1	5.0	11.7

^aGPDH activity is micromoles of NAD⁺ reduced per minute per milligram of protein in extracts of thoraces.

^bAldolase activity is micromoles of NADH oxidized per minute per milligram of protein in extracts of thoraces. Each enzyme activity value is a mean of three determinations.

that after four washes of the myofibril preparations GPDH is no longer found localized in the myofibrils, but an amount equivalent to the starting content of GPDH can be found in these four supernatants. That is why we have taken the steps to dissect muscle in fixative to obtain the myofibrils shown in Figure 4. Therefore, we believe the micrographs reveal the location of majority of the GPDH. Additionally, we think it unlikely that a major fraction of these enzymes has gone undetected in our biochemical assays. However, we recognize that our results present a static picture, and the mechanisms by which glycolytic enzymes provide energy for muscle contraction may involve dynamic processes that might include movement of these enzymes along the sarcomere.

We have shown that glycolytic enzyme colocalization is interdependent, in that localization of GAPDH and aldolase at the Z-discs and M-lines requires the presence of at least GPDH at the Z-discs and M-lines. Several interpretations of these findings with regard to mechanisms involved in colocalization are possible. First, these and presumably other glycolytic enzymes may participate in the formation of a structural complex at Z-discs and M-lines by means of direct contact with GPDH already localized at the Z-discs and M-lines. Therefore, in the absence of properly localized GPDH, participation of the other glycolytic enzymes in complex formation is precluded. A second possibility is that a glycolytic complex may form and then be localized to the Z-discs and M-lines using GPDH as a targeting molecule. Alternatively, formation of a complex of glycolytic enzymes in flight muscles may require a complete set of components for proper assembly and localization at the Z-discs and M-lines. Possibly, absence of any single component might disrupt the formation, targeting, or stability of such a complex. Models based on any of these interpretations may or may not involve specific association, involving contact between specific enzymes.

Our studies have been carried out exclusively on *Drosophila*. But there is a rather long history of studies using vertebrate cells that have generated proposals for the existence of glycolytic enzyme complexes.

Therefore, what we have described in *Drosophila* may be representative of a widely distributed phenomenon. Our results showing that glycolytic enzymes are colocalized in a pattern with the primary site being Z-discs and M-lines is parallel to a set of studies on creatine kinase in vertebrates (Turner *et al.*, 1973; Walilman *et al.*, 1977a,b) and the invertebrate analogue of creatine kinase, arginine kinase, in *Drosophila* (Lang *et al.*, 1980). These enzymes are also related to ATP metabolism (Van Waarde *et al.* 1990). Each has a pattern of localization in muscle cells essentially identical to the pattern of colocalization of GPDH-1, aldolase, and GAPDH that we have found in *Drosophila* flight muscle. These and other studies suggest a functional coupling between glycolytic enzymes and phosphocreatine metabolism. This gives additional support to the concept that the machinery necessary to produce ATP used for muscle contraction is highly organized, possibly in a dynamic manner. The mechanisms through which these enzymes become organized, their dynamic interactions, and the mechanisms that support the production of ATP for use as a substrate for myosin ATPase are of considerable interest. Genetic approaches have not been commonly used for studying glycolytic enzyme function, localization, or complex formation in higher eukaryotes, because these enzymes are usually essential for viability. However, modern genetic engineering approaches should enable studies on the role of enzyme organization that supports ATP production in muscle.

Studies on glycolytic complexes in vertebrates have generally fallen into three classes, each using biochemical criteria to show that a given glycolytic enzyme can associate with a specific partner. First, several studies have demonstrated that one or another glycolytic enzyme is found associated with, bound to, or coisolated with a second glycolytic enzyme partner (Srivastava and Bernhard, 1986a,b; Tompa, *et al.*, 1986; Srere, 1987; Kvassman *et al.*, 1988; Batke, 1989; Maughan and Wegner, 1989). Second, other studies have provided evidence that glycolytic enzymes associate with cytoskeletal components (Pierce and Philipson, 1985; Masters *et al.*, 1987; Mejean *et al.*, 1989; Knull and Walsh, 1992). Finally, others have demonstrated association of glycolytic enzymes with myofibrillar proteins (Lynch and Paul, 1983; Masters *et al.*, 1987; Roberts and Somero, 1987; Offer, *et al.*, 1988; Harris and Winzor, 1989; Mejean *et al.*, 1989; Masters, 1989). However, because these structural studies have not been done in conjunction with functional tests, it is not yet generally accepted that enzymes of glycolysis function as an organized complex, and controversy remains. Resolution of part of this controversy may reside in the possibility that some cell types contain glycolytic enzymes organized as complexes, whereas in other cell types these enzymes may be free in solution in the cytoplasm. In addition, glycolytic enzyme complex formation could be subject to physiological regulation, result-

ing in a fraction of the enzymes being in a complex under some physiological conditions but not others.

The possibility that glycolytic enzymes form complexes has been used to support the hypothesis that an organized complex of these enzymes contributes an important aspect to their function by providing the structural basis for a metabolic channel (Srivastava and Bernhard, 1986a,b; Masters *et al.*, 1987; Srere, 1987; Ovadi, 1988; Ovadi and Srere, 1992). This hypothesis has remained controversial (Kvassman *et al.*, 1988; Batke, 1989; Brooks and Storey, 1991; Wu *et al.*, 1991; Knull and Walsh, 1992), because it has been difficult to connect glycolytic enzyme complex formation directly with a specific function related to glycolysis in any one experimental system. We have shown that glycolytic enzymes are colocalized in flight muscle cells, that localization of one enzyme depends on the localization of another, and that colocalization is required for flight. We have not as yet demonstrated the existence of a glycolytic complex, but this seems a most likely extension of our data. If such a complex exists, it could provide the basis of a metabolic channel to maximize energy generation for flight muscle activity by affecting the rate of ATP production. An alternative possibility is that glycolytic enzyme colocalization provides a mechanism for compartmentalization of ATP pools. In any case, it is clear that a specific pattern of organization of these glycolytic enzymes is required for muscle function to support flight in *Drosophila*.

Our finding that a C-terminal tripeptide, Q-N-L, is necessary for GPDH-1 localization is reminiscent of targeting signals used in two other systems. These are peroxisomal targeting, which occurs in a variety of organisms (Gould *et al.*, 1990), and the targeting of glycolytic enzymes to the glycosome in trypanosomes (Oppenheimer, 1988). This latter membrane-bound subcellular organelle contains all of the glycolytic enzymes, including GPDH. In both cases, a C-terminal tripeptide is required for enzyme targeting of at least some of the enzymes of these organelles. Amino acid sequence requirements for targeting have recently been reviewed (Sommer and Wang, 1994), and it is apparent that there is similarity in the sequences used in each case. The consensus sequence for targeting is S-K-L, and the rules seems to be: first, a small amino acid; a penultimate amino acid that has a high probability of helix participation; and at the C-terminus, a hydrophobic amino acid, usually leucine. The C-terminal tripeptide of GPDH-1 is Q-N-L. Although Q is not a "small" amino acid, the others do conform to the consensus rules. It may be that mechanisms of protein targeting have an ancient evolutionary history. If so, some divergence might be expected if the targets are in organisms now as unrelated as trypanosomes and *Drosophila*. It is not yet known whether either GPDH-2 or GPDH-3 is localized at specific sites in the cells in which they are present. Interestingly, the terminal

three amino acids of GPDH-2 in the 10-amino acid domain encoded by isoform-specific exon 7 are P-K-L. This sequence is also highly reminiscent of the peroxisome- and glycosome-targeting signal.

ACKNOWLEDGMENTS

Dr. Judith Saide (Boston University, Boston, MA) generously provided antibodies to α -actinin, and Dr. Dean Tolan (Boston University) generously provided antibodies to aldolase. We thank Ross MacIntyre, David Turner, and Philip Dunham, who offered suggestions regarding the manuscript. This work was supported in part by National Institutes of Health grant GM-26830 (D.T.S.).

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