Concentrations of Glycolytic Enzymes and Other Cytosolic Proteins in the Diffusible Fraction of a Vertebrate Muscle Proteome*

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We used a novel microvolumetric technique based on protein diffusion to characterize the subproteome of muscle that consists of diffusible proteins, including those involved in cell metabolism. Muscle fiber segments were mechanically demembranated under mineral oil and transferred into drops of relaxing solution. After the fiber segment was depleted of diffusible proteins, the content of each drop and residual segment was analyzed by one-dimensional polyacrylamide gel electrophoresis. Proteins were identified through peptide mass fingerprinting and quantified using purified protein standards. Ten of the most abundant cytosolic proteins, distinguished by their ability to readily diffuse out of the skinned fiber, were glycolytic enzymes whose concentrations ranged from 2.6 ± 1.0 g liter⁻¹ (phosphoglucose isomerase) to 12.8 ± 1.1 g liter⁻¹ fiber volume (pyruvate kinase). The concentrations of the other five most abundant cytosolic proteins were as follows: glycogen phosphorylase, 6.0 ± 2.3 g liter⁻¹; phosphoglucose mutase, 2.2 ± 0.2 g liter⁻¹; adenylate kinase, 1.6 ± 1.3 g liter⁻¹; phosphocreatine kinase, 6.6 ± 2.6 g liter⁻¹; and parvalbumin, 0.7 ± 0.4 g liter⁻¹. Given the molecular weight and subunit number of each enzyme, the combined concentration of the 15 most abundant cytosolic proteins was 82.3 g liter⁻¹; the volume fraction was 0.093. The large volume fraction of diffusible proteins favors nonspecific interactions and associations, particularly if the glycolytic enzymes and diffusible phosphocreatine kinase are restricted to the I-band as previous studies suggest. The relative molar concentration of glycolytic enzymes is roughly consistent with a stoichiometry of 1:2 for enzymes catalyzing the hexose and triose sugar reactions, respectively, a stoichiometry that may favor metabolic channeling of intermediates during glycolysis. Our results indicate that subcellular fractionation of muscle proteins, in which cytosolic constituents are distinguished by their ability to diffuse readily from demembranated cells, is a promising microvolumetric technique that allows conclusions to be drawn about native protein-protein interactions based on concentration and stoichiometry. Molecular & Cellular Proteomics 4:1541–1549, 2005.

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The advent of proteomics, the experimental approach to assess global protein function, has introduced new techniques for studying important subproteomes of cellular proteins that operate in a coordinated fashion. One such subproteome is the entire complement of metabolic enzymes found in a selected muscle cell at any given time. Among these enzymes are those involved in glycolysis, a process by which ATP is generated during the enzymatic breakdown of glucose to pyruvate.

Glycolysis is important in fast twitch muscles where ATP, produced at intermediate steps of the glycolytic pathway, fuels contraction and a variety of other ATP-dependent cellular processes. Information about the subcellular distribution, concentrations, and mutual interactions of the glycolytic enzymes and their metabolites is important for a complete understanding of metabolism in fast twitch muscles.

In vertebrate fast twitch skeletal muscle fibers the glycolytic enzymes are regarded as soluble components of the fluid myoplasm because they are present in the supernatant fraction recovered after homogenization and centrifugation of the tissue sample (1). However, this assignment has often been a matter of dispute because the supernatant fraction may be only a rough approximation of the cytosol in situ (2–4). Not only are proteins in dilute slurries isolated under conditions far different from those in intact cells, additional information about their interactions may be lost using conventional methods of fractionation. Enzyme-enzyme interactions constitute an area of intense interest in structural genomics and enzymology (5, 6). Clearly studies in these areas would benefit from new approaches for isolation of proteins and determining their concentrations and stoichiometric relationships under conditions more closely approximating their native state. This information could help settle some controversial points debated over the past 50 years, such as whether large supramolecular complexes exist for glycolytic enzymes in the native cytosol (for a summary, see Ref. 5).

Warmke et al. (7) pioneered a microvolumetric method of subcellular protein enrichment and fractionation in which cytosolic constituents of muscle were distinguished on the basis of their ability to diffuse readily from a demembranated muscle fiber. This report describes the first step in carrying out a quantitative analysis of the diffusible protein fraction of a vertebrate muscle proteome using this method. We focus on glycolytic enzymes, proteins that constitute the major fraction
of vertebrate fast twitch muscle cytosol. Rabbit skeletal muscle was studied because purified standards of all the glycolytic enzymes and other muscle proteins of interest were commercially available for this experimental animal. Rabbit skeletal muscle has also been widely used for histochemical localization studies (8, 9) and enzymatic assays (10, 11) (see also Table III of Ref. 9 and citations therein), allowing a basis for comparing results. The diffusible proteins were separated by conventional one-dimensional polyacrylamide gel electrophoresis. Because the molecular weights and/or mobility of specific glycolytic proteins are similar to those of other muscle proteins, MALDI-TOF mass spectrum analysis was used to validate the content of the protein bands. Our approach provides a means to distinguish cytosolic proteins on the basis of their ability to diffuse readily from isolated, demembranated muscle cells bathed in physiological solutions closely approximating the ionic conditions within the cells. The concentrations and stoichiometry of the glycolytic enzymes are consistent with the hypothesis that they exist as mixed oligomers or multienzyme complexes in muscle cytosol that serve to channel glycolytic intermediates.

MATERIALS AND METHODS

Solutions—To avoid exposing the demembranated muscle cell to unusual ionic and osmotic conditions during fractionation, the bathing medium was designed to closely replicate the protein-free composition of vertebrate muscle cytosol (12). Accordingly the solution contained 6 mM MgCl₂, 5 mM Na₂ATP, 20 mM imidazole, 5 mM EGTA, 45 mM dipotassium creatine phosphate, 50 mM sucrose, and 4% (w/v) dextran T500 where ionic strength of the solution was 174 mM and the pH was 7.4 at 7 °C. Imidazole was added as a substitute for carnosine, which, in addition to bicarbonate, is the primary soluble proton buffer in muscle cytosol (12). Sucrose was added as an osmotic agent to balance the osmotic pressure across sarcoplasmic reticulum and mitochondrial membranes to preserve their integrity (13). The long-chain polymer dextran T500 (molecular mass, 500 kDa; Amersham Biosciences) was added to osmotically constrain the otherwise swollen skinned fiber and myofilament lattice, thus maintaining their in vivo size: i.e. the fiber diameter measured initially in oil (14).

Protein Standards and Gel Electrophoresis—Purified lyophilized proteins (Sigma) were used as known standards to (i) provisionally identify proteins on the basis of their electrophoretic mobility and molecular weight, (ii) corroborate the identity of selected proteins identified by mass spectrometry, and (iii) calibrate the amount of protein in determining concentrations. We carried out one-dimensional SDS-polyacrylamide gel electrophoresis with large format gels (10 × 10 cm) for optimum protein separation. Gels consisted of a 15% separating gel and 3.5% stack gel (200:1 and 20:1 acrylamide to bis ratio, respectively; pH 9.3 or 8.8; 0.75 mM Tris (15)). Sample buffer contained 62.5 mM Tris (pH 6.8), 1% (w/v) SDS, 0.01% (w/v) bromphenol blue, 15% (v/v) glycerol, and 15 mM dithiothreitol. Protein bands were silver-stained (as in Ref. 15), and their densities were quantified using a laser densitometer (model CS-930, Shimadzu Scientific Instruments, Columbia, MD). Densitometry of bands of purified proteins of equal loads indicated that all had similar stain affinities.

Skinned Fiber Preparation—Male New Zealand White rabbits (3–3.5 kg) were anesthetized via intraperitoneal injection of sodium pentobarbital and exsanguinated by decapitation. The surface of the psoas muscle was exposed by blunt dissection. Small bundles of fiber segments (1–3-cm length) were removed, cleansed in saline, blotted lightly, and placed in a glass-bottomed dish containing water-saturated mineral oil maintained at 7 ± 1 °C by a thermoelectric device. Single fiber segments were separated from the whole bundles, cut to 1-cm length, and stretched to 1.3 × slack length, and their diameter and length were measured using a filar micrometer and binocular dissecting microscope. Stretched fibers assume circular cross-sectional areas that are not significantly different from actual areas (16); therefore, fiber segment volumes were approximated by π/4 × diameter squared × length.

Fiber segments with the largest diameters were returned to their slack length and mechanically skinned (17) using needles to remove the sarcolemma and some underlying myofibrils. The amount of material removed was <5% initial volume. The segments were cut cleanly at each end to 5-mm length, and widths were measured (28.3–65.7 μm; mean, 43.7 μm; n = 39). Representative segments were photographed using a 35-mm camera attached to a compound microscope with 40× objective (Inverstoscope D, Zeiss, Thornwood, NY). Striation spacing measured from photomicrographs averaged ~2.2 μm.

Experiments were carried out at 7 °C rather than at body temperature to reduce metabolic activity and to maintain a stable preparation. At 7 °C skinned fibers remained translucent and fully relaxed, whereas at physiological temperature and pH (39.4 °C and 6.8 (18)) fiber segments became cloudy and eventually contracted. In preparing solutions, pH was adjusted to 7.4 to compensate for the temperature drop (preliminary tests indicated solution pH rises to 7.4 from 6.8 when solutions were cooled to 7 °C).

Separation of Proteins into Diffusible and Non-diffusible Fraction—Fig. 1 illustrates the fractionation method. Proteins were separated into diffusible and non-diffusible fractions by transferring a skinned segment under oil to a 10-μl drop of relaxing solution and allowing cytosolic proteins to diffuse out (the diffusible fraction). After 20-min equilibration (facilitated by occasional stirring), the segment was removed (the non-diffusible fraction) and sonicated in 20 μl of SDS sample buffer. Half of the solution drop containing the diffusible fraction was combined with an equal amount of 2× SDS sample buffer. Samples were in stored in capped plastic vials at −20 °C for further analysis.

Concentrations—To measure the concentration of diffusible protein, samples were thawed and diluted by various proportions to achieve an optimal load of ~5 ng per protein band in the sample gels. Optimal dilutions were determined by scanning sample gels and comparing band densities with those obtained from 5-ng loads of each purified protein standard. Protein standards were combined and sequentially diluted, and calibration curves were generated by loading the various dilutions on lanes adjacent to that containing the diffusible protein sample (19). The calibration curves indicated the linear range was −1–25 ng, similar to that obtained previously for parvalbumin (19). Concentrations, relative to whole fiber volume (g liter⁻¹ fiber), were calculated by dividing protein amount by fiber segment volume determined as above.

Enrichment of Protein Fractions and Tryptic Digestion for Peptide Mass Fingerprinting—Mass spectrometry was used to identify proteins in bands from gels loaded with enriched protein fractions. To provide enough material for peptide mass fingerprinting, bundles of muscle fiber segments were used. Proteins were separated into diffusible and non-diffusible fractions under oil as above except the fiber bundles were treated with 20 μg ml⁻¹ saponin (Sigma) to chemically (20), rather than mechanically, skin the fibers. At this concentration, saponin has a specific perforating action on the surface membrane (20). To facilitate diffusion, fibers were partially teased apart to increase diffusion surface area, and the solution droplet was stirred periodically. Proteins were separated via one-dimensional SDS-polyacrylamide gel electrophoresis using a 12% separation gel and a 5%
stack gel. Bands stained with Coomassie Blue were excised from gels with a scalpel, cut into smaller pieces, and placed in individual wells of a Montage In-gel Digestion kit digest plate (Millipore, Billerica, MA). Gel pieces were destained for 2 h in 100 μl of destain solution (25 mM ammonium bicarbonate in 50% acetonitrile) with several solution changes. Gel pieces were covered in 200 μl of 100% acetonitrile for 20 min and incubated with trypsin overnight at 30 °C. Digested peptides were extracted from the gel pieces and eluted via centrifugation. Samples (1.25 μl) were spotted on a stainless steel MALDI sample plate, dried, and treated with 1 μl of 2,5-dihydroxybenzoic acid (10 μg/ml in 100% methanol) applied to the top of each sample spot.

MALDI-TOF Mass Spectrum Analysis—Samples were analyzed in reflectron mode with a Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, CA). The following standard conditions were applied: accelerating voltage, 20 kV; grid, 75%; guide wire, 0.002%; delay time, 175 ns; shots/spectrum, 50; mass range, 10–3000 Da; and low mass gate, 500 Da. Three point calibrations were taken for each spot: MRFA (524.26 Da), bradykinin (1060.56), and angiotensin (1296.68). Data were acquired approximately five times per sample, 50 shots per acquisition.

All data sets were analyzed using the mass spectrometer software (Data Explorer, version 4.0, Applied Biosystems). Each spectrum was processed to eliminate background, improve offset baseline, and enhance signal-to-noise ratio (21). This procedure included noise filtering, background smoothing, and truncation of the spectrum to enhance signal-to-noise ratio (21). This procedure included noise filtering, background smoothing, and truncation of the spectrum to improve signal-to-noise ratio (21). This procedure included noise filtering, background smoothing, and truncation of the spectrum to enhance signal-to-noise ratio (21).

Protein Identification—The mass list of each sample was entered into the ProFound Peptide Mapping search form (prowl.rockefeller.edu) and used to query the National Center for Biotechnology Information (NCBI) non-redundant sequence database (version January 1, 2004 (28,089 sequences) through August 1, 2004 (27,913 sequences)) for single protein identification. Standard settings included: Taxonomy, other Mammalia; Enzyme, trypsin; Missed Cleavages, one; Modifications, partial methionine oxidation; Protein Mass, ranges entered when applicable to location on gel; Charge State, MH+ (monoisotopic); and Mass Tolerance, 0.1–1.0 Da, depending on the sample.

After searching the database, the website produced a ranked register of potential protein identifications (hits) that included an estimated Z-score (an indicator of the quality of the search result) for each sample. A Z-score corresponds to the percentile of the search in the random match population for all proteins entered into the database being searched. A successful identification was achieved with a Z-score in the 95th percentile or greater (i.e., 5% of random matches yield higher Z-scores than the search).

RESULTS

We identified glycolytic enzymes and other prominent diffusible cytosolic proteins obtained by microvolumetric fractionation (Fig. 1) by comparing their mobilities with those of purified protein standards (Fig. 2 and Fig. 3, lane 4). A 20-min incubation in relaxing solution was sufficient to deplete a typical ~45-μm-diameter skinned muscle fiber of diffusible proteins (Fig. 3, lane 3). All standards, except aldolase, produced single bands, indicating homomeric subunit composition and no appreciable contamination from other proteins. The electrophoretic mobility of each subunit was inversely proportional to its reported molecular weight as expected. Aldolase produced a doublet, which probably reflects single residue differences between two sets of subunits (22). The mix of protein standards exhibited extra bands above glycer- aldehyde-3-phosphate dehydrogenase (36.2 kDa), aldolase (39.2 kDa), and adenylate kinase (21.6 kDa), and lane 17 is parvalbumin (11.9 kDa). Lanes 3–17, 16 ng of protein loaded per lane; lanes 2 and 18, 10 ng.

Peptide mass fingerprinting was used to confirm the identity of the glycolytic enzymes and other diffusible proteins in enriched cytosolic fluid fractions obtained from bundles of chemically skinned muscle fibers (Fig. 1 and Fig. 3, inset). Table I lists Z-score and percent coverage of the proteins.
Fig. 3. Proteins separated by 15% polyacrylamide gel used for quantification of the cytosolic proteins and silver-stained. Lane 1, all proteins from whole fiber segment; lane 2, non-diffusible proteins; lane 3, diffusible proteins; lane 4, purified protein standards (as in Fig. 2). Capital letters (right side) indicate glycolytic enzymes. Shown are the contents of fractions from ~2 nl of fiber segment loaded per lane. Lane positions are rearranged to facilitate comparison. All labeled proteins were identified by peptide mass fingerprinting except tropinin-C (square brackets), each of which was provisionally identified by comparing its mobility to that of a purified protein standard (not shown). Inset, proteins from the cytosolic enrichment fraction separated by 12% polyacrylamide gel electrophoresis and Coomassie Blue-stained. The protein bands correspond to the boxed region of the 15% gel. The load was diffusible contents of ~200 nl of fiber bundles.

| Protein | EC | Swiss-Prot accession no. | Subunit molecular mass | Number of subunits | Z-score | Coverage |
|---------|----|--------------------------|------------------------|---------------------|---------|----------|
| Glycogen phosphorylase | 2.4.1.1 | P00489 | 97,158 | 2 | 2.39 | 29 |
| Phosphofructokinase | 2.7.1.11 | P00511 | 85,072 | 4 | 2.28 | 32 |
| Phosphoglucone isomerase | 5.3.1.19 | Q9N1E2 | 62,615 | 2 | 2.36 | 27 |
| Phosphofructokinase | 2.7.1.11 | P00511 | 85,072 | 4 | 2.28 | 32 |
| Phosphoglucone isomerase | 5.3.1.19 | Q9N1E2 | 62,615 | 2 | 2.36 | 27 |
| Pyruvate kinase | 2.7.1.40 | P11974 | 57,916 | 4 | 2.33 | 25 |
| Enolase | 4.2.1.11 | P25704 | 46,937 | 2 | 2.05 | 24 |
| Phosphocreatine kinase | 2.7.3.2 | P00563 | 43,112 | 2 | 2.31 | 23 |
| Aldolase | 4.1.2.13 | P00883 | 39,211 | 4 | 2.40 | 40 |
| Lactate dehydrogenase | 1.1.1.27 | P13491 | 36,433 | 4 | 2.40 | 47 |
| Glyceraldehyde-3-phosphate dehydrogenase | 1.2.1.12 | Not available | 36,175 | 4 | 1.98 | 23 |
| Phosphoglycerate kinase | 2.7.2.3 | Not available | 36,500 | 1 | 2.25 | 49 |
| Phosphoglycerate mutase | 2.7.1.40 | P00939 | 28,500 | 2 | 2.38 | 20 |
| Triose-phosphate isomerase | 5.3.1.1 | P00939 | 28,500 | 2 | 2.38 | 20 |
| Adenylate kinase | 2.7.4.3 | P00569 | 21,638 | 1 | 1.66 | 33 |
| Parvalbumin | 2.7.4.3 | P00569 | 21,638 | 1 | 1.66 | 33 |

* From Ref. 40.
* From Ref. 41.
* From Ref. 42.
The general absence of diffusible proteins in the non-diffusible fraction suggests the 20-min incubation was sufficient to clear the fiber segment of diffusible proteins. However, small residual amounts of phosphofructokinase and pyruvate kinase did remain in some depleted single fiber segments (<10% of the readily diffusible fraction), consistent with the larger size (Table I) and putative lower diffusivity of these proteins compared with the others.

In overloaded samples, a small amount of myosin appeared in the diffusible fraction (0.5–1% of that in the non-diffusible fraction) of both single fiber segments and chemically skinned bundles. This small diffusible fraction probably represents an upper limit to the monomeric pool of myosin in equilibrium with filamentous myosin. Our inability to detect diffusible counterparts of other cytomatrix proteins in overloaded samples from both assays suggests that the critical concentration for protein polymerization or complex formation of other cytomatrix proteins is below that of myosin.

Table II lists the concentrations of 10 glycolytic enzymes calculated from measurements made in the single fiber segment assay. Values range from 2.6 g liter\(^{-1}\) (phosphogluco-

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**Table II**

| Enzyme                        | No. of fibers | Concentrations (mean ± S.D.)\(^a\) |
|-------------------------------|--------------|-------------------------------------|
|                               |              | Fiber volume                        |
|                               |              | g liter\(^{-1}\) \(\mu\)mol liter\(^{-1}\) |
| Phosphoglucone isomerase      | 23           | 2.6 ± 1.0                           |
| Phosphofructose kinase        | 23           | 8.4 ± 1.0                           |
| Aldolase                      | 23           | 6.0 ± 2.1                           |
| Triose-phosphate isomerase    | 22           | 3.1 ± 1.5                           |
| Glyceraldehyde-3-phosphate dehydrogenase | 19 | 7.6 ± 0.5                           |
| Phosphoglycerate kinase\(^c\) | 23           | 6.8 ± 2.5                           |
| Phosphoglycerate mutase       | 22           | 2.8 ± 0.7                           |
| Enolase                       | 22           | 7.2 ± 1.5                           |
| Pyruvate kinase               | 23           | 12.8 ± 1.1                          |
| Lactate dehydrogenase\(^c\)   | 23           | 7.9 ± 2.8                           |

\(^a\) Grand mean and S.D. of averages from at least four rabbits (at least four samples each) unless otherwise indicated.

\(^b\) Molar concentrations correspond to oligomeric forms of the proteins calculated on the basis of the number of subunits and subunit molecular masses listed in Table I. Cytosolic fluid volume is defined as fiber volume minus the volume of cytomatrix proteins and intracellular organelles. The factor 1/0.71 converts mol g liter\(^{-1}\) fiber volume to mol liter\(^{-1}\) fluid (cytosolic) volume outside the sarcoplasmic reticulum, mitochondria, and cytomatrix proteins (32). Mole of protein per liter of fiber volume (mol liter\(^{-1}\) fiber) equals gram of protein per liter of fiber volume (g liter\(^{-1}\)) divided by gram of protein per mole (i.e., molecular weight). Molecular weights are from Table I.

\(^c\) Mean and S.D. of eight samples from one rabbit. Total concentration of aldolase, phosphoglycerate kinase, and diffusible phosphocreatine kinase is 19.4 g liter\(^{-1}\) of which 0.31 (≈6.01 g liter\(^{-1}\)) is aldolase and 0.35 (≈6.79 g liter\(^{-1}\)) is phosphoglycerate kinase (see text).
Concentrations of Diffusible Muscle Proteins

**Table II**

Concentrations of five other abundant cytosolic proteins whose values range from 0.7 g liter$^{-1}$ (parvalbumin) to 6.6 g liter$^{-1}$ (phosphocreatine kinase). Although bands constituting aldolase, phosphoglycerate kinase, and diffusible phosphocreatine kinase often overlapped in the gel system used for quantification (Fig. 3), these proteins were usually well resolved in the gel system used for peptide mass fingerprinting (Fig. 3, inset). Assuming equal staining characteristics, the latter provided a measure of the fractional abundance of aldolase (0.31 ± 0.01), phosphoglycerate kinase (0.34 ± 0.01), and diffusible phosphocreatine kinase (0.35 ± 0.02), whereas the former provided a measure of the total concentration of all three proteins (19.4 ± 6.8 g liter$^{-1}$). Taken together, these measures yielded the concentration estimates for aldolase, phosphoglycerate kinase, and diffusible phosphocreatine kinase listed in Tables II and III.

**DISCUSSION**

**Identifications**—The goal of this study was to identify and measure the concentrations of the major diffusible proteins constituting the cytosolic subproteome of rabbit psoas muscle. Through the use of peptide mass fingerprinting, we identified all of the expected glycolytic enzymes in the cytosolic enrichment fraction plus glycogen phosphorylase, phosphoglucose mutase, phosphocreatine kinase, adenylate kinase, and parvalbumin. By introducing a quantitative method to characterize a subproteome of muscle proteins based on diffusivity, we advance previous efforts to characterize aspects of the vertebrate muscle proteome (9, 24), efforts that have culminated in current broad based proteomic studies designed to identify isofrom switches and post-translational changes that accompany aging and disease (see e.g. Refs. 25 and 26). Of the diffusible fraction, nearly all proteins identified were expected constituents of the cytosol (9, 12, 24). Surprisingly ADP/ATP translocase, an abundant mitochondrial protein (27), was also detected in the cytosolic enrichment fraction. Although the presence of diffusible ADP/ATP translocase suggests mitochondrial contamination (despite the relatively low abundance of mitochondria in fast twitch fibers), the fact that other mitochondrial proteins were not detected raises the possibility that ADP/ATP translocase is indeed a diffusible enzyme. If so, it may serve a role in shuttling MgADP to mitochondria and MgATP to myofibrils through facilitated diffusion. ADP/ATP translocase has been found to be a constituent of the myofibrillar fraction in *Drosophila* indirect flight muscle (28), suggesting this enzyme may play a similar role in the metabolism of insect flight muscle.

**Concentrations**—The list of concentrations of the major diffusible proteins in rabbit psoas muscle (Tables II and III) confirms that proteins involved in cell metabolism are the most abundant. Concentrations of the glycolytic enzymes (Table II) range from 2.6 g liter$^{-1}$ fluid volume (phosphogluco-

cose isomerase) to 12.8 g liter$^{-1}$ (pyruvate kinase). The concentrations of phosphoglucose mutase (2.2 g liter$^{-1}$) and glycogen phosphorylase (6.0 g liter$^{-1}$), listed in Table III, are similar to those of the glycolytic enzymes, consistent with a coupling of expression of these metabolic enzymes. The high concentrations of adenylate kinase (1.6 g liter$^{-1}$) and phosphocreatine kinase (6.6 g liter$^{-1}$), also listed in Table III, are not surprising because these enzymes play an important role in buffering MgATP and fueling contraction in fast twitch skeletal muscles (29–31). The presence of the soluble calcium-binding protein parvalbumin (Table III) is consistent with the well established role of parvalbumin in facilitating relaxation in fast twitch muscles (32). However, its concentration (0.7 g liter$^{-1}$) is only a tenth of that found in frog semitendinosus muscle (7.4 ± 0.8 g liter$^{-1}$ (19)), suggesting that its role in relaxing fast twitch skeletal muscles may be less important in rabbits than it is in frogs (32).

The concentrations listed in Tables II and III are generally higher, after conversion to grams per kilogram wet weight, than those reported elsewhere for rabbit fast twitch skeletal muscle. Fig. 4 compares present values with those reported by Scopes (24) and Pette (9), which are, as far as we can determine, the most recent and comprehensive surveys of their kind. Most of the concentrations determined in the current study are at least a factor of 2 greater than those listed in both studies. The reasons for the discrepancies are unclear. Although the muscle type in the present study was not the same as that used in previous studies, all starting material was rabbit fast twitch muscle. Likewise, although individual fiber segments may not necessarily represent the whole muscle, it is unlikely that the composition of the fibers varies so greatly within one muscle as to account for the large differ-
the fact that the length of the thick filament (length given a fluid volume fraction of 0.076 (sum of proteins in Table II plus phosphocreatine in Table III). The factor 1/0.25 is derived from (volume fraction, 0.087; as calculated in Table IV). However, extremely crowded (2, 34).

Table IV legend), supports the view that myoplasm is ex-

ularly in the region bounded by the Z-disc and A-band (see

ume fraction of diffusible proteins in the fiber cytosol, partic-

has a similar pattern of localization (29). Thus, the large vol-

zarcomere (for citations, see Ref. 33). Phosphocreatine kinase

zymes are not distributed uniformly throughout the cytosolic

ences between studies. Most likely, the discrepancies arise from differences in sample preparation and analysis. Values reported previously, using a variety of enzymatic assays, are derived from supernatant fractions after homogenization and centrifugation of the muscle samples. It is possible, therefore, that significant amounts of protein were either not recovered or enzymatic activities were blunted during the recovery pro-

cedures. Both problems would contribute to reducing the concentration estimates. Thus we conclude that the values listed in Tables II and III are more likely to reflect the true concentrations because they are derived from simple, con-

current measurements of diffusible protein concentration from structurally preserved cell segments.

Volume Fractions, Molar Concentrations, and Enzyme As-

sociations—Given the concentration of proteins in the cyto-

solic fraction, what can one conclude about the state of the cytosol and the interactions between proteins? The combined concentration of all 15 diffusible proteins listed in Tables II and III is 82.3 g liter⁻¹ or ~1.1 mmol liter⁻¹ fiber fluid. These 15 proteins represent nearly 9% of the cytosolic fluid in the fiber (volume fraction, 0.087; as calculated in Table IV). However, in situ immunohistochemical studies show that glycolytic en-

zymes are not distributed uniformly throughout the cytosolic fluid; rather they are localized to the I-band region of the sarcomere (for citations, see Ref. 33). Phosphocreatine kinase has a similar pattern of localization (29). Thus, the large vol-

ume fraction of diffusible proteins in the fiber cytosol, partic-

ularly in the region bounded by the Z-disc and A-band (see Table IV legend), supports the view that myoplasm is ex-

remely crowded (2, 34).

There is considerable circumstantial evidence that glyco-

lytic enzymes form associations in crowded conditions (1, 2,

33–37) (summarized in Table III of Ref. 5 and in Ref. 38). Although direct evidence for glycolytic enzyme complexes remains elusive, our results suggest a tantalizing stoichiometric relationship of 1:2 for the hexose:triose glycolytic enzymes (Fig. 5) that is consistent with a "metabolic channeling" func-

TABLE IV

Estimate of volume fraction of 15 most abundant diffusible proteins in rabbit skeletal muscle

| Symbol | Parameter | Value | Ref. |
|--------|-----------|-------|------|
| C      | Total concentration of diffusible proteins | 82.3 g protein liter⁻¹ fiber | Tables II and III |
| ρ      | Average specific density of protein per liter protein | 0.75 kg liter⁻¹ | 45 |
| α      | Volume of cytosolic fluid per fiber volume* | 1.41 liter fluid liter⁻¹ fiber | 46 |
| v_F   | Volume fraction of diffusible proteins in fiber | 0.087 | v_F = C × ρ × α |

* Cytosolic fluid volume does not include the mitochondria or sarcoplasmic reticulum fluid volumes or the volume of solids constituting the cytomatrix and organellar proteins. Assuming that glycolytic enzymes (33) and the diffusible fraction of phosphocreatine kinase (29) are confined to the I-band region of the sarcomere, the I-band volume fraction of these proteins is 0.304 (=0.076 × 1/0.25) at 2.2-μm sarcomere length given a fluid volume fraction of 0.076 (sum of proteins in Table II plus phosphocreatine in Table III). The factor 1/0.25 is derived from the fact that the length of the thick filament (i.e. the width of the A-band) is 1.6 μm and the width of the Z-disc is 0.05 μm (43); thus, the fractional width of the I-bands at 2.2-μm sarcomere length is 0.25 = (2.2 μm - 1.6 μm - 0.05 μm)/2.2 μm. Based on Fig. 4, we speculate that glycolytic enzymes form a complex in the I-band of one molecule of each enzyme in the hexose pathway and two molecules of each enzyme in the triose pathway. The mass of the complex is 2.094 MDa, the volume is 4 × 10³ nm³, and the (spherical) diameter is ~20 nm, assuming subunits of average density 1.33 g cm⁻³ and a solid volume fraction of 0.64 for random close packing (44) of proteins and their subunits (see text “Discussion”).

Fig. 5. Concentrations (in micromoles per liter fiber fluid) and stoichiometric relationships of glycolytic enzymes from rabbit psoas muscle. Dashed lines indicate grand mean of enzymes in hexose (39 ± 13 μM) and triose (88 ± 23 μM) pathways. The group differences suggest the triose pathway requires twice as many molecules of the rate-limiting enzyme as the hexose pathway to accommodate an additional flux of intermediates.

Molecular & Cellular Proteomics 4.10 1547
phoglycerate mutase, pyruvate kinase, and lactate dehydrogenase forming each putative complex. Assuming the specific activities of the rate-limiting enzymes in the hexose and triose pathways are similar (which, in \textit{vitro}, is \textit{not} the case under dilute conditions \textsuperscript{1}), the triose pathway would require twice as many molecules of the rate-limiting enzyme as the hexose pathway to accommodate the additional flux of intermediates.

The 1:2 stoichiometry of the putative complex differs from that of a 12-enzyme complex proposed by Kurganov et al. \textsuperscript{38} that contains one molecule of pyruvate kinase and two molecules each of the other glycolytic enzymes plus one molecule of phosphoglucone mutase and one of fructose-1,6-biphosphatase. Although the projected mass of each complex is large (2.094 and 2.86 MDa, respectively), a volume calculation (Table IV legend) suggests that complexes of this size can be accommodated within the 18–20-nm surface-to-surface filament spacing of the I-band lattice if the complexes are somewhat elongated rather than spherical or if the filaments themselves are flexible enough to bend around them. Indeed I-band structures appear to bridge the gap between neighboring filaments in chemically fixed, freeze-fractured, or freeze-substituted frog and insect striated muscles. It is possible these so-called “I-bridges” may be assemblies of glycolytic enzymes because they are absent in fibers that have been skinned and incubated in relaxing solution, in which soluble contents were allowed to diffuse out under experimental conditions similar to those in this study. \textsuperscript{39}

\textbf{Summary and Implications}—Subcellular fractionation of proteins in which cytosolic constituents are distinguished by their ability to diffuse readily from demembranated cells is a promising microvolumetric technique that allows conclusions to be drawn about native cytosolic protein-protein interactions based on concentration and stoichiometry. In the present study the relative concentrations of glycolytic enzymes in the diffusible fraction of rabbit psoas muscle suggest a stoichiometry of 1:2 for enzymes catalyzing the hexose and triose sugar reactions, respectively. The large volume fraction of diffusible proteins in the fiber cytosol supports the view that the myoplasm is very crowded, particularly in the region bounded by the Z-disc and A-band. The resulting enzyme-enzyme interactions presumably determine the final enzyme ratios that optimize metabolic channeling of intermediates during glycolysis.

Despite past successes in localizing proteins like the glycolytic enzymes and phosphocreatine kinase to the I-band, the nature and extent of their physical interactions remain elusive. Part of the reason may be that the putative associations are dynamic and not easily pinned down. During sarcomere shortening, large complexes must dissociate so as not to hinder cross-bridge formation or thick and thin filament sliding. However, the complexes must also reassociate and remain stable within the time frame of glycolysis. Such dynamic associations are probably exquisitely sensitive to the local ionic environment, further complicating our attempts to isolate and characterize them.
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