Molecular Evolutionary Convergence of the Flight Muscle Protein Arthrin in Diptera and Hemiptera

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Uniquely, the asynchronous flight muscle myofibrils of many insects contain arthrin, a stable 1:1 conjugate between actin and ubiquitin. The function of arthrin is still unknown. Here we survey for the presence of arthrin in 63 species of insect across nine orders using Western blotting. Analysis of the evolutionary distribution shows that arthrin has evolved a limited number of times but at least once in the Diptera and once in the Hemiptera. However, the presence of arthrin does not correlate with any observed common features of flight mechanism, natural history, or morphology. We also identify the site of the isopeptide bond in arthrin from *Drosophila melanogaster* (Diptera) and *Lethocerus griseus* (Hemiptera) using mass spectrometry. In both species, the isopeptide bond is formed between lysine 118 of the actin and the C-terminal glycine 76 of ubiquitin. Thus, not only the ubiquitination of actin but also the site of the isopeptide bond has evolved convergently in Diptera and Hemiptera. In terms of the actin monomer, lysine 118 is near neither the binding sites of the major actin-binding proteins, myosin, tropomyosin, or the troponins, nor the actin polymerization sites. However, molecular modeling supports the idea that ubiquitin bound to an actin in one F-actin strand might be able to interact with tropomyosin bound to the actin monomers of the other strand and thereby interfere with thin filament regulation.

Introduction

Arthrin, found in the thin filaments of the asynchronous flight muscles of many insects, is stably ubiquitinated actin (Ball et al. 1987). Estimates of the arthrin:actin ratio vary from 1:3 (Sous et al. 1984) to 1:6 (Ball et al. 1987). Arthrin migrates slower on denaturing gels than expected from its mass, suggesting that the ubiquitin is conjugated by an isopeptide bond to an internal lysine (Ball et al. 1987). Support for this hypothesis came recently from observations that the actin ubiquitination does not occur at the N-terminus (Schmitz et al. 2000). Three-dimensional reconstructions from electron micrographs of in vitro polymerized F-arthrin showed that ubiquitin is located on subdomain 1 of the actin monomer on the reverse side from the tropomyosin binding sites for both *Drosophila melanogaster* (Burgess et al. 2000) and the hemipteran *Lethocerus indicus* (Galkin et al. 2003). By fitting atomic structures of actin and ubiquitin into their reconstructions Galkin et al. (2003) speculate that the ubiquitin is most likely conjugated to lysine 118. During myogenesis, ubiquitination of actin occurs several hours after actin synthesis and polymerization of actin into thin filaments (Ball et al. 1987). In vitro, F-arthrin can be decorated with myosin S1 fragments. It activates the myosin ATPase and can be regulated by tropomyosin-troponin in the same ways as F-actin (Bullard et al. 1985). A limited characterization of actomyosin kinetics using stop-flow methods also showed no difference between arthrin and actin (Schmitz et al. 1998). The function of arthrin in the myofibril therefore remains completely unknown.

Polyubiquitination is a common and essential step in the selective degradation of many intracellular proteins (Hershko et al. 1984). Indeed, in vitro actin too can be degraded by the ubiquitin-dependent pathway (Mayer et al. 1989). However, very few proteins form stable ubiquitin conjugates. The best studied of these are the histones, where reversible ubiquitination may affect histone function (Nickel, Allis, and Davie 1989) and thereby modulate gene regulation (Sun and Allis 2002). Reversible monoubiquitination of calmodulin modulates its biological activity (Laub et al. 1998). Further, stably ubiquitinated proteins are the herpes simplex virus type 1 proteins ICPOR (Weber, Spatz, and Nordby 1999) and US9 (Brandimarti and Roizman 1997). Apart from asynchronous insect flight muscles, stably ubiquitinated actin has also been found in the merozoites of the malaria parasite *Plasmodium falciparum* (Field et al. 1993). Neither the function nor the ubiquitination site of the parasite actin are known. An immunohistochemical study of many breast tumor samples revealed the much increased presence of an ubiquitinated protein in all tumors investigated (Iwaya et al. 1997). Upon proteolytic digestion, the protein produced many peptides identical to sequences within the two human cytoplasmic actin isoforms. It remains unclear if the ubiquitinated protein, with an apparent mass of only 43 kDa is (partially degraded?) actin or a new protein of the actin family.

Arthrin may play a role in the unusual structural and functional properties of asynchronous insect flight muscles. Bullard et al. (1985) and Ball et al. (1987), for example, speculate on an involvement of arthrin in stretch activation, a characteristic property of these muscles (reviewed by Dickinson and Tu 1997) in which both calcium and a passive stretch are required for full muscle activation. The similar stoichiometry of arthrin and the tropomyosin-troponin thin filament regulatory complex (troponin:actin ratio is 1:7) has led a number of authors (Bullard et al. 1985; Ball et al. 1987; Peckham et al. 1992) to speculate on a functional interaction between arthrin and

Key words: actin, ubiquitin, arthrin, evolution, isopeptide bond.

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Mol. Biol. Evol. 20(12):2019–2033. 2003
DOI: 10.1093/molbev/msg212
Molecular Biology and Evolution, Vol. 20, No. 12,

tropomyosin-troponin. In that respect, the presence of troponin-H in asynchronous insect flight muscles, an unusually large form of troponin unknown in other types of muscles (Bullard et al. 1988), may be of importance. Troponin-T in *D. melanogaster* has an acidic tail similar to that of the ubiquitin-conjugating enzyme RAD6, and Peckham et al. (1992) speculate that the proximity of ubiquitin to an acidic tail in the flight muscle may have some unknown function similar to that in the ubiquitin-conjugating system. The actin of asynchronous insect flight muscles is, at least in *D. melanogaster*, apart from its ubiquitination to form arthrin, also unusual in another way. It is the only actin known with an unacetylated and thereby free N-terminus (Schmitz et al. 2000).

Peckham et al. (1992), investigating the potential relationship between stretch activation and arthrin, examined flight muscle samples from 20 species from eight orders of winged insects. Arthrin was present in the asynchronous flight muscles of seven species from two orders, Diptera and Hemiptera, but absent from all synchronous flight muscles. It was also absent in many of the asynchronous flight muscles, including those of the primitive dipteran *Tipula*. From the last observation, the authors concluded that arthrin cannot be necessary for stretch activation but might still modulate stretch activation when present. In this study, we have extended the work of Peckham et al. (1992) to look for arthrin in a further 63 species of winged insects, covering a variety of different branches of the evolutionary tree. The first goal was to reconstruct the evolutionary history of this protein modification. The second goal was to compare the presence of arthrin with any obvious common features of flight mechanism, natural history, or insect morphology. Arthrin was identified by its apparent mass on SDS-PAGE gels and reaction with an antiactin antibody, similar to the approach chosen by Peckham et al. (1992). Results were additionally confirmed for some species with an anti-ubiquitin antibody. Our third goal was to identify the sites of the isopeptide bonds in arthrin for *D. melanogaster* and *L. griseus* by purifying the proteins and using mass spectrometry to determine the peptides containing the isopeptide bonds. The final goal was to learn about the molecular structure of arthrin using molecular modeling to seek a function for arthrin within the thin filament.

Materials and Methods

Evolutionary Distribution

Trapping, Identification, and Storage of Insects

Most insects were trapped in Malaise traps (70% ethanol, emptied weekly) on the University of York campus in summer 2001 and stored in 100% ethanol at 4°C. The moths were caught in a mercury vapor trap in a garden near York in the same season and stored as above. The *L. griseus* was obtained for us by M. Reedy (Duke University) from Florida. The insects were identified as far as possible to species (especially Lepidoptera) and always to family, except for one caddisfly, one thrip, one booklouse, and one earwig. These were the only representatives of their respective orders. For family identifications, we used Oldroyd (1970) (Diptera), and Gauld and Bolton (1988) (Hymenoptera), otherwise Chinery (1986). Species identification of moths was achieved using Skinner (1998).

Dissection and Homogenization of Thoraces

Thoraces were dissected under a light microscope by removal of the head with watchmaker's forceps, and the abdomen, wings, and legs were cut away with microscissors. Five thoraces for each species were then immersed in an appropriate volume of York Modified Glycerol (YMG: 20 mM KP_i buffer pH 7.0, 1 mM NaN_3, 1 mM DTT, 2 mM $MgCl_2$, 50% [v/v] glycerol, 1% [v/v] Triton X-100 [White 1983]) in 1.5 ml Eppendorfs and stored at least overnight at –20°C. The thorax samples stored in YMG were pelleted by centrifugation at 13,000 rpm in a benchtop centrifuge for 5 min, and the supernatant discarded. An appropriate volume (depending on the size of the thoraces between 200 and 1000 µl) of 1× homogenization buffer (HB) containing Triton X-100 as a detergent to remove membrane proteins was added (100 mM NaCl, 10 mM KPi, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 0.1% [w/v] trypsin inhibitor pH 7.0, supplemented with 0.5% [v/v] Triton X-100), the sample homogenized using a Kontes homogenizer and pelleted at 13,000 rpm for 5 min. This was repeated twice with HB containing Triton X-100 and a further three times using HB only. The final supernatant was discarded and the samples were resuspended in an appropriate volume (between 30 and 150 µl) of 1D SDS-PAGE sample buffer (10% [v/v] glycerol, 5% [v/v] 2-mercaptoethanol, 2% [w/v] SDS, 0.002% bromophenol blue [w/v], 63 mM Tris-Cl pH 6.8). To denature the proteins, the samples were heated to 90°C for 5 min.

Western Blotting

1D SDS-PAGE was carried out according to the method of Laemmli (1970) using the BioRad Mini-Protean II gel electrophoresis system with 12% (w/v) acrylamide. Before loading, the samples were centrifuged for 5 min at 13,000 rpm, and 15 µl of the supernatant were loaded for each sample. Duplicate gels were run for all the samples; one was analyzed for the presence of proteins by Coomassie staining (not shown), the other was Western blotted for the presence of actin and arthrin. Gels were soaked for 40 min in transfer buffer (25 mM Tris pH 8.3, 150 mM glycine 20% methanol), and the proteins were transferred to nitrocellulose filters at 250 mA for 75 min using the BioRad Mini Trans-Blot Electrophoretic transfer cell. The filters were washed in TBS (20 mM Tris pH 7.5, 0.15 M NaCl, 0.01% Thimerosal, 0.2% Tween-20) for 20 min, incubated in blocking buffer (TBS and 5% Marvel™ milk powder) for 2 h at 4°C and again washed in TBS for 20 min. For binding of the primary antibody, the filters were incubated in a 1/50 dilution of the rabbit anti–ACT88F-specific antibody (courtesy of M.-C. Lebart, University of Montpellier) in TBS for 2 h. This antibody was specifically raised against the first 10 N-terminal amino acids of the *D. melanogaster* indirect flight muscle–specific ACT88F actin isoform. For some species, samples were run on additional gels Western blotted for the

presence of arthrin using a rabbit antiubiquitin primary antibody at a dilution of 1/20 (Sigma U5379). Unbound primary antibody was washed off by five washes with TBS for 10 min. For hybridization with the secondary antibody, the filters were incubated in a 1/1000 dilution of the goat horse radish peroxidase conjugated anti–rabbit IgG secondary antibody (Sigma) in TBS for 2 h. The bound secondary antibody was visualized using an enhanced chemiluminescence kit (Amersham) according to the manufacturers instructions and recorded by exposure to autoradiograph film (Fuji RX) for between 5 s and 1 min.

Trees

A composite cladogram was constructed for those taxa investigated. Orders were clustered according to a figure 20 in Wheeler et al. (2001), who conducted the most recent comprehensive estimate of hexapod phylogeny. Their review incorporates all the morphological and molecular evidence that can be compared for a large proportion of taxa. For the taxa included here, the estimate is noncontroversial and widely accepted. The dipteran phylogeny was constructed thus: major subgroups were clustered according to Friedrich and Tautz (1997). This molecular study has resolved four major groupings previously suggested by different taxonomists to represent monophyletic groups: the Brachycera, Bibionomorpha *sensu lato*, Tipulomorpha *sensu stricto*, and Culicomorpha, with Psychodidae and Trichoceridae unplaced. For Orthorhapha, we followed Woodley (1989) and Wiegmann, Mitter, and Thompson (1993), and for Cyclorrhapha, we followed McAlpine (1989). For the Hemiptera, we placed the Sternorrhyncha as the outgroup to Auchenorrhyncha + Heteroptera, following a number of authors, and for Auchenorryncha, we followed the classification of Carver, Gross and Woodward (1991), clustering Cercopidae with Cicadellidae. Heteroptera were clustered thus: (Gerridae (((Nepidae, Belostommatidae), Notonectidae), (Pyrrhocorridae, (Miridae, (Nabidae, Anthocoridae))))) following Schuh (1979), Wheeler, Schuh, and Bang (1993), Schuh and Stys (1991), and Schuh and Slater (1995). The only critical phylogenetic assumption we made was that the Nepomorpha are a monophyletic group, since within this group, arthrin was present, but outside this froup, arthrin was absent. Clustering within other orders was similarly unimportant, since there was no variability within orders, and we followed accepted classifications throughout. The resulting composite cladogram contained several soft polytomies, representing areas of uncertainty in the phylogenetic tree. The most influential of these was between the dipteran subgroups as defined by Friedrich and Tautz (1997). In the analysis, we investigated the effect of this uncertainty by generating trees from the base tree by randomly resolving the polytomies to create fully bifurcating trees.

Analysis

We coded each of the terminal taxa in the data set as either containing arthrin, not containing arthrin, or equivocal. To reconstruct the history of arthrin evolution, we used Fitch parsimony (Fitch 1971) implemented in MacClade version 3.0 (Maddison and Maddison 1992), assigning equal cost to gain and loss of arthrin. The parsimony algorithm distils information from all parts of the tree surrounding any node by taking as its solution those states occurring in the greatest number of three sets of most-parsimonious solutions for a node; those from the left and right descendents and those from the ancestor. Consistency and character retention indices were also calculated (Farris 1969, 1989). The consistency index measures how close the number of reconstructed character steps is to the minimum possible on any tree (as a proportion where 0 = infinitely more and 1 = minimum) and is therefore a measure of homoplasy. Since we have only two character states and a single character, the minimum possible steps is one. The retention index measures whether the observed number of steps is closer to the minimum or maximum possible on any tree (as a proportion where 0 = maximum and 1 = minimum) and is therefore a measure of how readily characters reverse their states. MacClade 3.0 was also used for random resolution of polytomies. A sample is drawn at random from the population of fully dichotomous trees that are consistent with the polytomous one, assigning equal probability to each possible tree.

Identification of the Isopeptide Bond in Arthrin from *D. melanogaster* and *L. griseus*

Protein Concentrations and Purity Assessment

Protein concentrations and purity were assessed by Coomassie stained 1D SDS-PAGE (see above) by visual estimation of the stain intensity of the proteins compared to BSA standards of known amounts.

Purification of Arthrin

With the minor modifications mentioned below, the method of Razzaq et al. (1999) was used to purify arthrin from whole-fly actin preparations by anion exchange chromatography. Five grams of actin acetone powder, prepared from 25 g of flies, yielded 5 ml of a whole-fly actin isoform mixture with a protein concentration of about 1.5 mg/ml. A 2-ml sample was applied to a 1-ml Mono Q (Pharmacia) anion exchange column. The arthrin was separated using a linear gradient from 0 to 350 mM NaCl in 20 mM Tris-HCl (pH 7.0) at a flowrate of 1 ml/min. Fractions of 250 µl were collected in 10× polymerization buffer (50 mM Tris, 500 mM KCl 20 mM $MgCl_2$, 10 mM ATP, pH 8.0), excess rhodamine-phalloidin was added, and the arthrin was polymerized overnight at 4°C. F-arthrin was pelleted at 100,000 rpm for 20 min at 4°C in a Beckman TLA100.4 rotor by consecutive spins of the arthrin fractions in a single centrifuge tube to combine all the arthrin in a single, larger, and therefore easier to handle pellet. The fluorescent rhodamine facilitated handling of the pellet by allowing visualization of the protein. Phalloidin increases the yield by reducing the critical concentration of the arthrin. Finally, the arthrin pellet was washed and taken up in 50 µl of the tryptic digest buffer (50 mM NH_4HCO_3, pH 7.8).

For the much bigger *L. griseus*, actin and arthrin were copurified from the dissected dorso-longitudinal indirect flight muscles of a single insect. The method is essentially a scaled down version of the one described above to purify the *D. melanogaster* actin isoform mixture from whole flies but with the minor modifications mentioned below. The dissected dorso-longitudinal muscles yielded more than 1 g of actin acetone powder, of which 0.3 g were extracted. After the KCl cut to remove the contaminating thin filament proteins, the resulting F-actin pellet (a mixture of indirect flight muscle actin and arthrin) was washed and directly taken up in 200 µl of the tryptic digest buffer (50 mM NH_4HCO_3, pH 7.8).

Denaturation and Tryptic Digest

The *D. melanogaster* arthrin and the *L. griseus* mixture of indirect flight muscle actin and arthrin were denatured by the method of Hoaglund-Hyzer and Clemmer (2001) for denaturing ubiquitin. The samples were heated for 20 min at 90°C and rapidly cooled to 0°C in an ice bath. For digestion, trypsin (12.5 mg/ml sequencing grade modified trypsin [Promega]) was added at a protease:protein ratio of 1:30 (w/w) and the samples were incubated for 20 h at 37°C. The samples were then frozen and kept at –80°C, and 20 µl aliquots were dried in a SpeedVac, washed twice with 100 µl of water to remove volatile salts, and resuspended in 20 µl 0.1% formic acid.

MALDI-ToF Mass Spectrometry for Peptide Mass Fingerprint

The tryptic peptide mixtures (0.5 µl) were spotted onto the MALDI target plate and mixed with 0.5 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA [Sigma]) in 50% acetonitrile/0.1% trifluoroacetic acid. The plate was then dried under a constant stream of warm air, loaded into the Voyager DE-STR mass spectrometer (Perseptive) and spectra acquired using the Perseptive Voyager version 5.0 software. The mass range between 500 and 4,500 Da was measured. Close external calibration was performed using the standard Perseptive peptide calibration mix 2. The baselines of the spectra were corrected, noise removal performed (SD = 2.0), and the peak list deisotoped (so that only the mass of the monoisotopic peptide peak was included in the list), using the Perseptive Data Explorer Software. Using a mass tolerance of 0.7 Da, the experimental masses of the 40 strongest peptide peaks were compared with theoretical peptide masses (see below).

Protein Sequences

For all the actins, numbering refers to the amino acid position in mammalian skeletal muscle actin aligned with the residue in that particular sequence. For the *D. melanogaster* arthrin, the sequences taken from Swiss-Prot at ExPASy Molecular Biology Server (http://www.expasy.ch/) were ACT88F = ACT6_DROME, P02575 actin and human ubiquitin (identical to *D. melanogaster* ubiquitin) = UBIQ_HUMAN, P02248. For the ACT88F actin, all known posttranslational modifications were considered (Schmitz et al. 2000). A theoretical tryptic digest of actin and ubiquitin was made using PeptideMass (http://ca.expasy.org/tools/peptide-mass.html) to predict the expected peptide sequences and masses. Peptide masses were calculated as protonated $[M+H]^+$ and monoisotopic, and no missed cleavage sites were allowed. Further theoretical masses needed for the calculations were taken from FindMod tool (http://www.expasy.org/tools/findmod/findmod_masses.html). Theoretical isoelectric points for tropomyosin (P09493), actin (P02575), ubiquitin (P02248), and troponin-I (P48788) mentioned in the discussion were calculated using the ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html).

As the protein sequences for the *L. griseus* arthrin were unknown, a different approach was chosen to match experimental with theoretical peptide masses. The peptide mass fingerprint (experimental masses of the 40 strongest peptide peaks) was searched against the Swiss-Prot database using PeptIdent (http://us.expasy.org/tools/peptident.html). The appropriate options were selected as described above (mass tolerance of 0.7 Da, peptide masses are $[M+H]^+$ and monoisotopic, no missed cleavage sites), limiting the search to insect actins. The best match was then taken as the sequence template for the actin part of the *L. griseus* arthrin. Unidentified peptide peaks were then individually matched with insect actins or ubiquitin by using the same database, search machine, and parameters. Newly identified peptides from other insect actins were then used to change the sequence template for the actin part of the *L. griseus* arthrin. PeptIdent automatically considers acetylation of the N-terminus as well as a free N-terminus for the actins in question. Finally, since almost all actins contain a methylated histidine at the equivalent of position 73 of vertebrate skeletal muscle actin (Sheterline, Clayton, and Sparrow 1998), this mass was looked for in the appropriate peptides.

A complete tryptic digest of arthrin will also produce a peptide having sequences of actin as well as of ubiquitin. Because of the 19 lysines of actin, this peptide will have one of 19 theoretical masses, which can be calculated as follows. Trypsin will not cut at the lysine forming the isopeptide bond. Therefore, this peptide will be one with a missed lysine cleavage site. With an arginine at position 74, the C-terminal tryptic fragment of ubiquitin consists of glycines 75 to 76. This fragment will also be part of such a peptide, as in the conjugation of ubiquitin to a target protein, the C-terminal amino acid of ubiquitin (glycine 76) provides the carboxyl group for the new peptide bond (Hershko et al. 1984). There is no mass difference between adding amino acids internally or via an isopeptide bond. The 19 masses calculated for *D. melanogaster* and *L. griseus*, respectively, were compared with the experimental masses. A matching actin fragment will contain an internal lysine, a possible candidate for the isopeptide bond. To confirm identification, that peptide was subjected to MS/MS to obtain sequence information (see below).

HPLC-ESI Mass Spectrometry with MS/MS for Peptide Sequencing

HPLC-ESI mass spectrometry with MS/MS was performed with a Finnigan LCQ-DECA mass spectrometer

coupled to an Agilent HP1100 HPLC system using the EC 250/2 Nucleosil 100-5 C18 PPN Column from Macherey-Nagel. For each of the two samples, 10 μl of proteolytic peptides were applied and eluted with a gradient of acetonitrile from 20% to 95%, in the presence of 0.1% HCOOH. Instrument control was obtained with the program Xcalibur (Thermoquest). General methods employed the "triple play" mode comprising full scan, zoom scan, and MS/MS of multiple charged ions. Dedicated HPLC-MS runs were performed with two focused scan events, one for a zoom-scan and one for the MS/MS experiment of the particular mass to be examined.

Molecular Modeling

Ubiquitin was docked onto the surface of an actin trimer. The docking was done interactively using the program molviewer-ogl (M. J. Hartshorn, personal communication; Caves et al. 1997). The docking procedure was as follows. Throughout the modeling, the three monomers of the actin protofilament were treated as a single rigid body. As a rigid body, ubiquitin was maneuvered to place its tail (residue 76) in close proximity to lysine 118 of one of the actin monomers. Thereafter, ubiquitin was partitioned in the following way. Residues 70 to 76 were treated as individual rigid bodies connected via a flexible phi bond (the torsion about C-Cα bond). The remainder of ubiquitin was treated as a single rigid body. Subsequently, interactive docking was executed in the following fashion. Residue 76 was fixed in space, the main body of the ubiquitin was allowed to translate without rotating, and the segmented ubiquitin tail was allowed to rotate and translate freely. The docking was performed interactively under the influence of a potential energy function, incorporating simple quadratic penalty functions to maintain bonded geometry and a nonbonded clash function, which disfavors poor steric interactions. The final docked geometry is to be regarded as illustrative of a sterically reasonable configuration only. No extensive optimization of the interaction between the ubiquitin and actin was performed, as the interactive modeling procedure finds the nearest local energy minimum of the simple energy function computed "on the fly" during the docking; important interactions such as electrostatics were not considered. The primary objective was to examine the bulk steric consequences of docking ubiquitin on the actin filament, tethered by its flexible tail region to lysine 118 on actin. The docking explored configurations in the approximate vicinity of the region that tropomyosin is thought to occupy in the presence of myosin (Vibert, Craig, and Lehman 1997). For computational efficiency, the docking was performed with only the N, Cα, and C atoms of the molecules. Figure 7 shows an illustrative docked configuration with solvent accessible surface areas computed for a probe size of 8 angstroms on a 2-angstrom grid.

Results

Evolutionary Distribution

The presence of arthrin was analyzed in 63 species of insects from nine orders. Figure 1 shows the Western blots

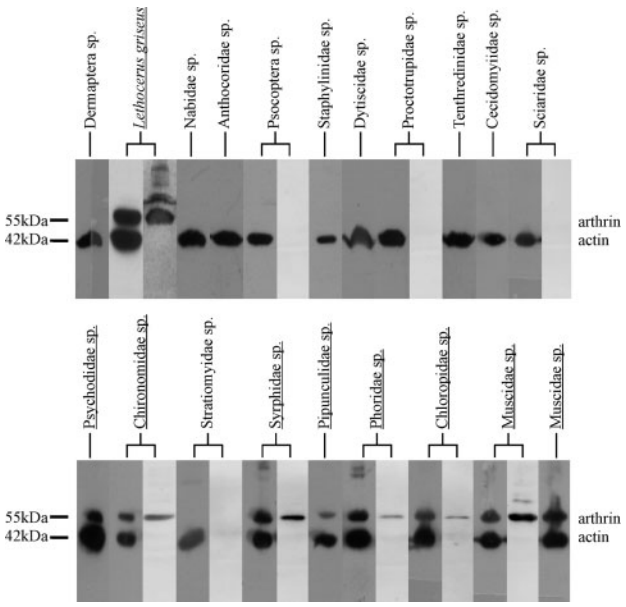


FIG. 1.—Western blots of 1D SDS-PAGE separations of thorax protein samples from insects from selected taxa. The band at about 42 kDa represents actin, and the band at about 55 kDa, where present, represents arthrin (underline indicates species scored for presence of arthrin). Where two lanes are shown for a species, the second lane shows antiubiquitin Western blots. For some species, these show unknown proteins of high molecular weight (see text for details). All other lanes are antiactin Western blots.

of several species, and figure 2 summarizes the results for all 63 species. The actin antibody shows a positive reaction at about 42 kDa, the expected size of actin, for all species. Actin therefore acts as a control for the antibody reaction in different species. Several species show an additional second positive reaction at about 55 kDa, the expected size of arthrin on gels. In these species, the 55 kDa band is weaker than the band at 42 kDa. In all cases, the 42 kDa and the 55 kDa bands (if present) were always the first and strongest reactions. Unspecific reactions could only be seen at increased exposure times.

For some selected species (see fig. 1), a ubiquitin antibody was additionally used to confirm that the upper band was arthrin. For those species where the actin antibody failed to detect arthrin, so did the ubiquitin antibody. The ubiquitin antibody showed a strong reaction at the expected size of arthrin for those species in which the actin antibody detected arthrin. These results show that the protein detected at about 55 kDa is indeed ubiquitinated actin. The ubiquitin antibody weakly stained some high-molecular-weight proteins in some insects, an observation also reported by Ball et al. (1987) for *D. melanogaster*. As these proteins do not react with the actin antibody, they are not related to actin.

Antibodies are used here primarily because of their high specificity to unequivocally confirm the presence or absence of arthrin. The very high sensitivity of the Western blotting method used is not necessarily required for the detection of arthrin in those species where the protein has been found here. For *D. melanogaster* for example, the indirect flight muscles from five flies contained about 8 μg of actin, representing about 30% of the

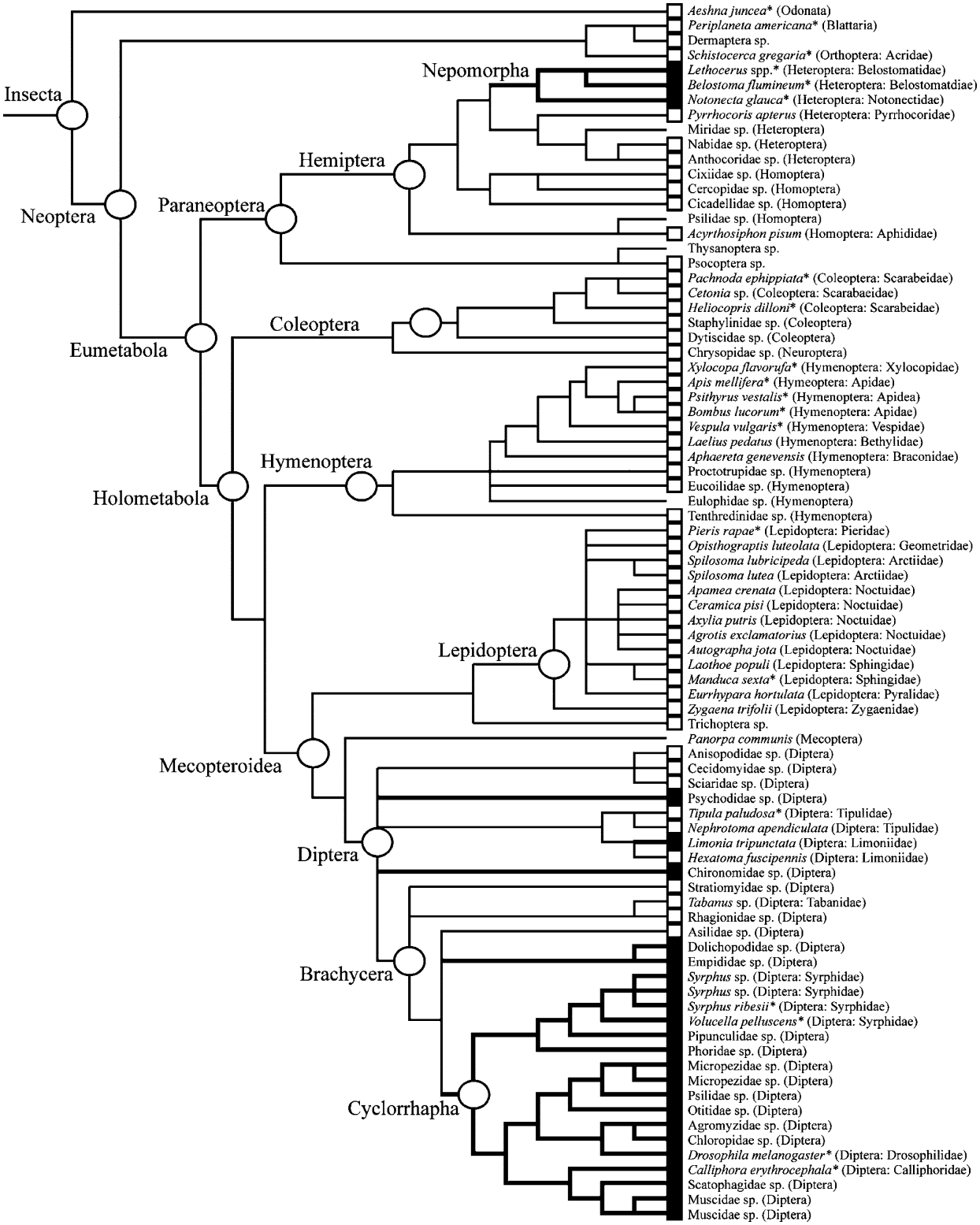


FIG. 2.—Phylogenetic tree for the occurrence of arthrin in the insect taxa surveyed (asterisk [*] indicates data from Peckham et al. [1992]) and for and the most parsimonious reconstruction of arthrin evolution (bold branches = arthrin presence; fine branches = arthrin absence) using the composite cladogram based on Wheeler et al. (2001) and others (see text). Filled boxes = arthrin presence, open boxes = arthrin absence, and no box = uncertain.

total myofibrillar protein (Ashman et al. 1997). With the ratio of arthrin:actin at about 1:6 (Ball et al. 1987), quantities of approximately 1 μg of arthrin were assayed with the antibodies, a level six orders of magnitude above the sensitivity of the detection reagent used (Amersham). The ratios of arthrin:actin observed in Western blots here confirm that where arthrin was detected, it was present in quantities similar to those expected of other muscle structural proteins (e.g., troponin-T [Bullard et al. 1985]) and not in enzymatic quantities. If arthrin were present in the species we scored as negative, it would be in very small and very different quantities compared with those in which the protein was detected. In those circumstances, it could not have a similar functional role.

Figure 2 lists the occurrence of arthrin in the taxa surveyed. The base tree contained a total of four character transitions. The ancestral state for all orders was absence of arthrin. There were two unambiguous gains of arthrin (in the Nepomorpha and *Limonia* [Diptera]) and no unambiguous losses. The consistency index was 0.25, and the retention index was 0.88. Some ambiguous changes were possible because of unresolved phylogeny or equivocal reconstruction. To see how many, 500 fully bifurcating trees were created by randomly resolving polytomies in the base tree. This led to a maximum of six and minimum of two gains of arthrin and a maximum of four and minimum of zero losses of arthrin. On average there were 3.54 unambiguous gains of arthrin and 0.02 unambiguous losses. The ambiguous gains and losses all occurred in the Diptera.

To test how parsimonious the conclusion of convergent evolution of arthrin in the two orders was, we observed how many evolutionary steps were required if a single evolution of arthrin were assumed. This scenario required 14 steps (i.e., 3.5 times as many steps as the most parsimonious solution), including five losses in the Paraneoptera, six losses in the Diptera, and three losses in other orders. If the presence of arthrin is assumed more simply to be homologous and plesiomorphic for the ancestral members of the orders Hemiptera and Diptera, then 12 steps are required, including five losses in the Paraneoptera, three losses in other nondipteran orders, and four changes in the Diptera. Thus, the scenario of convergent evolution in the Hemiptera and Diptera is very parsimonious.

Identification of the Isopeptide Bond in Arthrin from *D. melanogaster* and *L. griseus*

Purification of Arthrin

The charge separation (not shown) of the *D. melanogaster* whole-fly actin preparation by anion exchange chromatography resembled the one shown by Schmitz et al. (2000). The arthrin fractions yielded about 75 μg of arthrin taken up in 50 μl of the tryptic digest buffer (fig. 3), contaminated with only a small amount of actin. The copurification of actin and arthrin from the dissected dorso-longitudinal indirect flight muscles of *L. griseus* yielded about 400 μg of a mixture of actin and arthrin taken up in 200 μl of the tryptic digest buffer, with arthrin at a concentration of about 0.3 μg/μl (fig. 3).

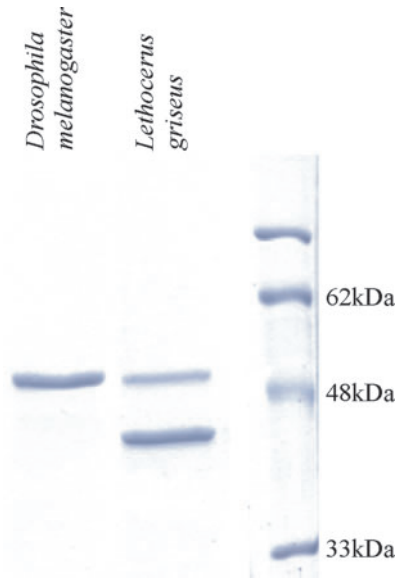


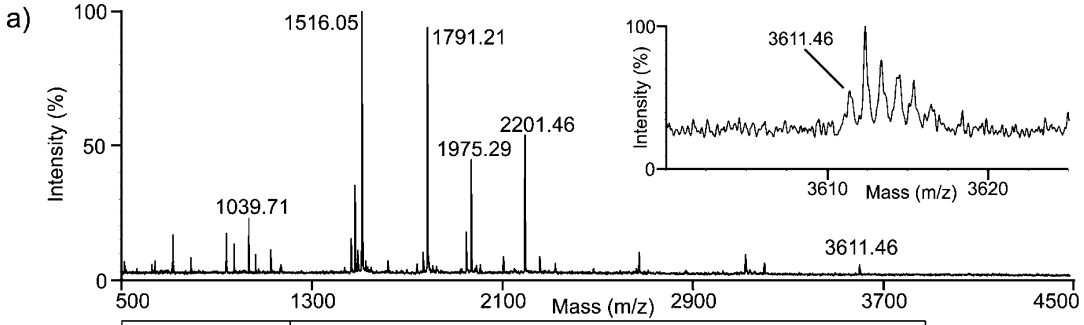
FIG. 3.—Coomassie stained 1D SDS-PAGE of purified arthrin from *D. melanogaster* and mixture of arthrin and actin from *L. griseus*.

MALDI-ToF Mass Spectrometry for Peptide Mass Fingerprint

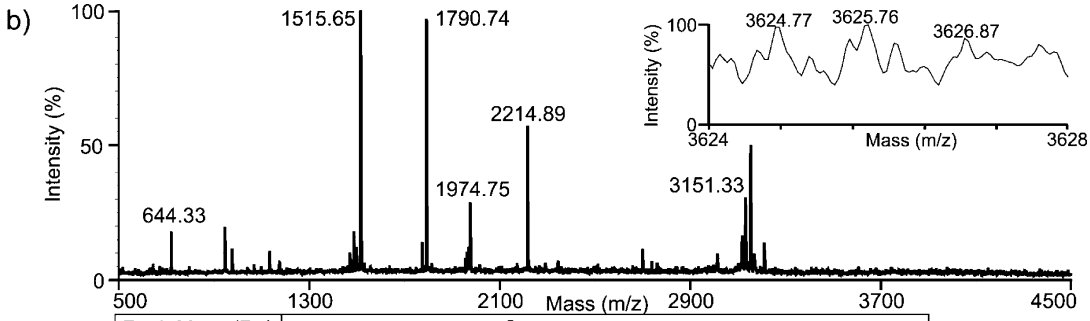
Figure 4*a* shows the MALDI-ToF mass spectra of the tryptic digest of arthrin from *D. melanogaster*. The table in figure 4*a* contains the 40 strongest deisotoped peaks of the full spectra for which matching theoretical masses were found. The matches found cover 54% of the actin sequence and 55% of the ubiquitin sequence. Only one of the experimental masses (3611.46 Da) matched one of the theoretical masses (3610.77 Da) for a peptide having both sequences of actin and ubiquitin. The inset in figure 4*a* shows a zoom-scan of the spectra at 3611 Da, corresponding to an actin fragment containing amino acids 117 to 147. Lysine 118 is therefore a possible candidate for the isopeptide bond in arthrin from *D. melanogaster*.

Figure 4*b* shows the MALDI-ToF mass spectra of the tryptic digest of the mixture of actin and arthrin from the flight muscles of *L. griseus*. The table in figure 4*b* contains the 40 strongest deisotoped peaks of the full spectra for which matching theoretical masses were found. The flight muscle actin sequence of *Bactrocera dorsalis* (Oriental fruit fly), ACT3_BACDO (P45886), was the best match against this peptide mass fingerprint. Two further matches were found, both peptides from the flight muscle actin sequence of *Bombyx mori* (Silk moth), ACT2_BOMMO (P07837).

Figure 5 shows the resulting sequence template for the actin part of *L. griseus* arthrin. The matches found cover 54% of that sequence template. Based on that sequence template, only one of the experimental masses (3,624.77 Da) matched one of the theoretical masses (3,624.79) for a peptide having both actin and ubiquitin sequences. The inset in figure 4*b* shows a zoom-scan of the spectra at 3,625 Da. The isotope pattern suggests, despite the low signal-to-noise ratio, that the peak at 3,624.77 Da indeed represents a true peptide of that mass. The respective actin fragment again includes residues 117 to



Peak Mass (Da)		Sequence
516.41	207-210	EIVR
631.51	192-196	ILTER
644.50	63-68 and/or 178-183	GILTLK and/or LDLAGR
717.48	49-54	QLEDGR
720.47	285-290	CDVDIR
795.62	329-335	IIAPPER
976.64	19-28	AGFAGDDAPR
1039.71	34-42	EGIPPDQQR
1067.82	64-72	ESTLHLVLR
1132.76	197-206	GYSFTTTAER
1486.97	360-372	QEYDESGPGIVHR
1516.05	85-95	IWHHTFYNELR
1788.22	12-27	TITLEVEPSDTIENVK
1791.21	239-254	SYELPDGQVITIGNER
1954.43	96-113	VAPEEHPVLLTEAPLNPK
1975.29	69-84(Met)	YPIEH(Met)GIITNWDDMEK
2201.46	292-312	DLYANSVLSGGTTMYPGIADR
2491.66	216-238	LCYVALDFEQEMATAAASTSLEK
3611.46	117-147 + 75-76	EK(GG)MTQIMFETFNSPAMYVAIQAVLSLYASGR



Peak Mass (Da)		Sequence
516.28	207-210	EIVR
631.34	192-196	ILTER
644.33	63-68 and/or 178-183	GILTLK and/or LDLAGR
720.28	285-290	CDVDIR
795.43	329-335	IIAPPER
976.39	19-28	AGFAGDDAPR
1039.43	34-42	EGIPPDQQR
1132.47	197-206	GYSFTTTAER
1486.60	360-372	QEYDESGPGIVHR
1515.65	85-95	IWHHTFYNELR
1790.74	239-254	SYELPDGQVITIGNER
1960.74	69-84	YPIEHGIITNWDDMEK
1967.90	96-113	*VAPEEHPILLTEAPLNPK
1974.75	69-84(Met)	YPIEH(Met)GIITNWDDMEK
2214.89	292-312	*DLYANTVLSGGTTMYPGIADR
2491.26	216-238	LCYVALDFEQEMATAAASTSLEK
3151.33	148-177	TTGIVLDSGDGVSHTVPIYEGYALPHAILR
3253.56	119-147	MTQIMFETFNTPAMYVAIQAVLSLYASGR
3624.77	117-147 + 75-76	EK(GG)MTQIMFETFNTPAMYVAIQAVLSLYASGR

FIG. 4.—Analysis of peptide mass fingerprints of arthrin by MALDI-ToF mass spectrometry. (*a*) Spectra (500 to 4,500 Da), zoom-scan at 3,611 Da, and peptide mass fingerprint of the tryptic digest of arthrin from *D. melanogaster*. Ubiquitin sequences are underlined. (*b*) Spectra (500 to 4,500 Da), zoom-scan at 3,625 Da, and peptide mass fingerprint of the tryptic digest of the mixture of actin and arthrin from the flight muscle of *L. griseus*. Actin sequences from the flight muscle actin sequences of *Bactrocera dorsalis* (Oriental fruit fly), ACT3_BACDO (P45886) and (*) *Bombyx mori* (Silk moth), ACT2_BOMMO (P07837). Ubiquitin sequences are underlined. All masses are experimental.

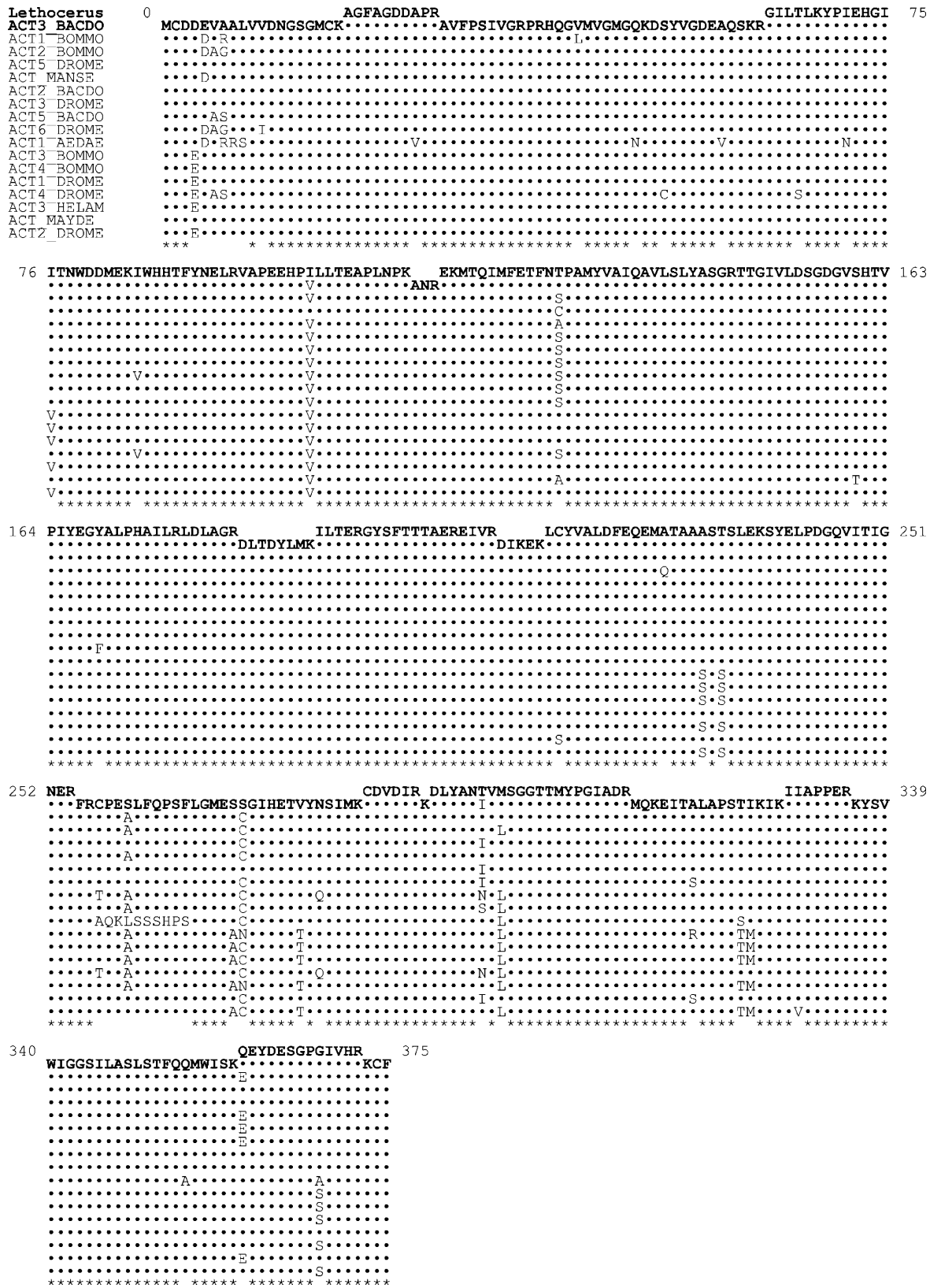


FIG. 5.—Alignment of the peptide mass fingerprint of the tryptic digest of the mixture of actin and arthrin from the flight muscle of *L. griseus* by MALDI-ToF mass spectrometry (Lethocerus, line 1) with all SWISS-PROT insect actins (asterisk [*] in last line indicates conserved residues within this alignment). Derived sequence template for the actin part of the *L. griseus* arthrin is sequence in the first line completed by the flight muscle actin sequence of *Bactrocera dorsalis* in the second line. The sequences were aligned with ClustalX version 1.8 (Thompson et al. 1997).

147. Lysine 118 therefore is also a possible candidate for the isopeptide bond in arthrin from *L. griseus*.

HPLC-ESI Mass Spectrometry with MS/MS for Peptide Sequencing

For unequivocal identification of the peptide with the mass of about 3,611 Da, the *D. melanogaster* arthrin peptide mix was separated by HPLC, and the masses of the eluting peptides were measured. ESI spectra usually show doubly protonated tryptic peptides, whereas MALDI mostly yields singly protonated species. A peptide with the m/z of 1,806.3 Da was found, with the expected m/z of 1,806.4 Da ([3,610.77 Da + 2 Da]/2 = 1,806.4 Da). Figure 6*a* shows a zoom scan of the peak, which is in accordance with the expected isotope distribution and mass of the peptide. The subsequent fragmentation within the mass spectrometer (MS/MS) resulted in a series of singly charged peptides. As expected (''Y-Series''), the charge is located on the C-terminal arginine, so that partial sequencing can be obtained from the relative mass differences of the MS/MS peaks. The sequence is identical to the 14 actin residues 129 to 142 of the tryptic fragment 117 to 147. The peptide containing actin as well as ubiquitin sequences is therefore unequivocally identified. In the *D. melanogaster* arthrin, the isopeptide bond is therefore formed between lysine 118 of actin and glycine 76 of ubiquitin. Methylation of histidine 73 of the actin was also proved by partial sequencing of the respective peptide (not shown).

To identify unequivocally the *L. griseus* peptide with the mass of about 3,625 Da, the arthrin and actin peptide mix was separated by HPLC, and the masses of the eluting peptides were measured. A peptide with the m/z of 1,813.2 Da was found, with the expected m/z of 1,813.4 Da ([3,624.79 Da + 2 Da]/2 = 1,813.4 Da). Figure 6*b* shows a zoom scan of the peak, which is in accordance with the expected isotope distribution and the mass of the peptide. The subsequent fragmentation within the mass spectrometer (MS/MS) resulted in a series of singly charged peptides. Again, partial sequencing was obtained from the relative mass differences of the MS/MS peaks and showed a sequence identical to the 13 actin residues 130 to 142 of the tryptic fragment 117 to 147. The peptide containing actin as well as ubiquitin sequences is therefore unequivocally identified. The isopeptide bond in *L. griseus* arthrin, as in *D. melanogaster*, is formed between lysine 118 of actin and glycine 76 of ubiquitin. Methylation of histidine 73 of the actin was again confirmed by partially sequencing the respective peptide (not shown).

Discussion

The number of insect species for which the presence or absence of arthrin has been reported is 83 (fig. 2), including 20 from an earlier study (Peckham et al. 1992). Arthrin was previously found in three species of Hemiptera (all Heteroptera: Nepomorpha) and four species of Diptera (all Cyclorrhapha), but it was known not to occur throughout the Diptera (it was absent from Tipula), and it has not been found in any other insect order (Peckham et al. 1992). In this paper, we have surveyed a further 63 taxa, bringing the total surveyed to 83 taxa. The new data have failed to extend the number of orders containing arthrin, suggesting that this represents the true ordinal extent. The data have also refined our knowledge of where it is found within those orders. In the Hemiptera, none of the new taxa, including several Heteroptera, contained arthrin, suggesting that it may be unique to the Nepomorpha in nondipterous insects. In the Diptera, we found arthrin in all the Cyclorrhapha surveyed (17 taxa in 12 families), in some but not all of the remaining Brachycera, and in some but not all of the other major dipteran subgroups (*sensu* Friedrich and Tautz 1997).

Parsimony analysis suggests that arthrin has evolved at least twice (once in each order) and possibly five times in the Diptera. It may also have been lost in some Diptera, depending on the precise resolution of the basal Dipteran subgroups. The earliest fossil records for Notonectidae and Belastomatidae, as well other Nepomorpha (e.g., Naucoridae), come from the Triassic Carnian, about 229.2 MYA (Benton 1993), which gives an approximate date for the first appearance of arthrin in the Hemiptera. In the Diptera, arthrin may have originated basally (i.e., 255.6 MYA [Benton 1993]) and, more conservatively, had arisen by the origins of the Cyclorrhapha in the Lower Cretaceous (~146 MYA [Hennig 1981; Benton 1993]). Thus, arthrin apparently arose in both groups sometime in the Mesozoic. The small number of evolutionary steps observed is reflected in the high retention index for the base tree and suggests that arthrin (or its absence) is evolutionarily relatively unlabile (phylogenetically conserved). Arthrin is however homoplastic; that is, it does display some convergent evolution and possibly some reversal among the insects as a whole. However, within the Hemiptera, it currently appears synapomorphous for the Nepomorpha, and its distribution within the major dipteran subgroups may indicate common ancestry. For instance, all the Cyclorrhapha and Bibionomorpha (*sensu lato*) surveyed possessed arthrin. We suggest, therefore, that arthrin may have some relevance in dipteran and heteropteran systematics.

The function of arthrin is currently unknown, and with only a limited number of evolutionary gains and loses, it is impossible for us to provide statistical support for any functional hypotheses through evolutionary correlations. However, our data do narrow the functional possibilities. For example, arthrin is still only found in insects with asynchronous flight muscle, the tissue from which it was originally detected (Bullard et al. 1985). Within the Hemiptera, asynchronous muscle is found throughout the Heteroptera and in some Sternorrhyncha (Cullen 1974; Pringle 1981), whereas arthrin is known only from the Nepomorpha (Heteroptera). The Diptera all have asynchronous muscle. Arthrin cannot be an essential component of asynchronous muscle, because it has not been detected in any Hymenoptera or Coleoptera, and it has not been detected in some Diptera, Heteroptera, and Sternorrhyncha, despite these groups having asynchronous muscle. Furthermore, the function does not appear to be correlated with any broad category of flight mechanism. For example, the Diptera are all two (fore) winged,

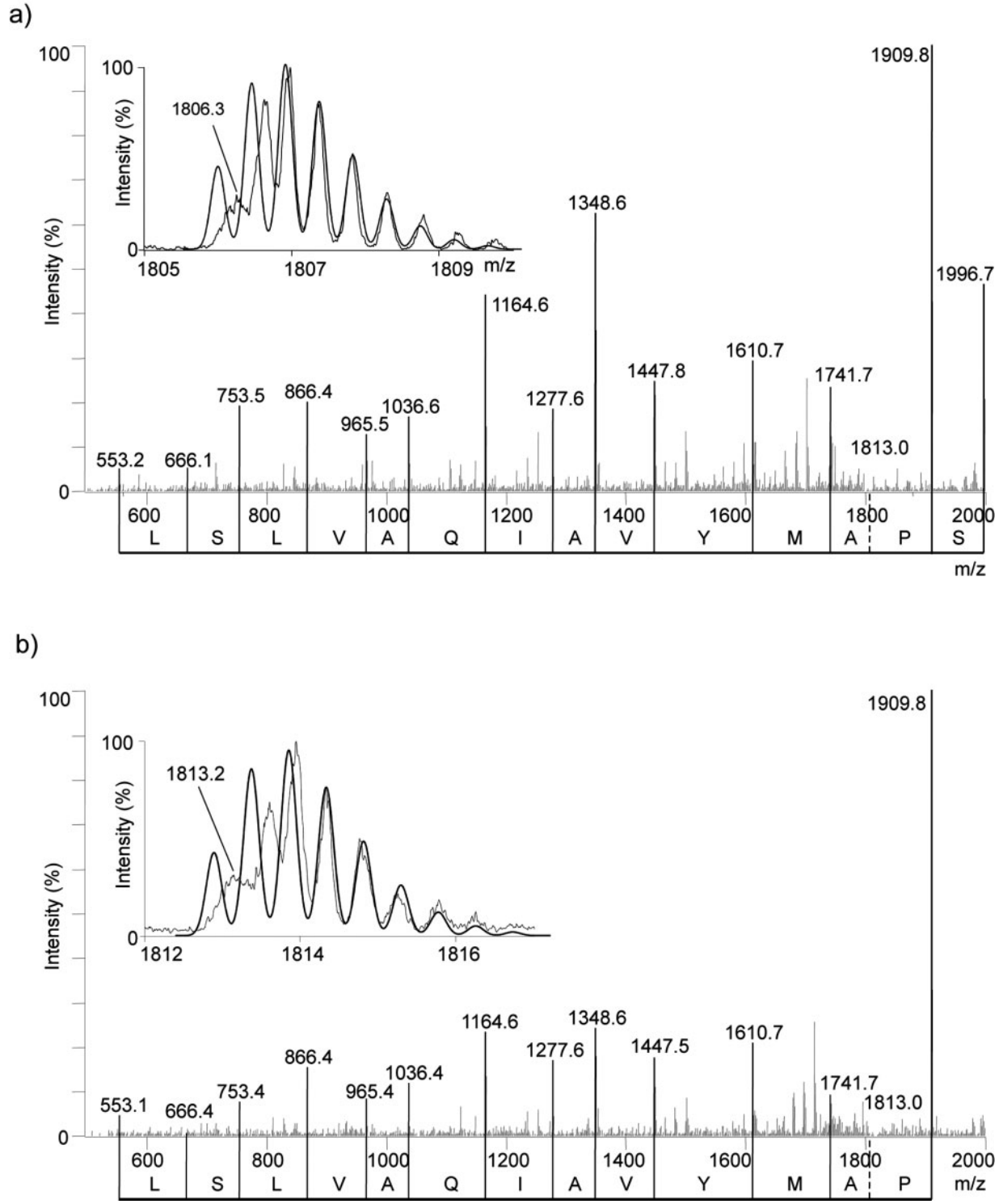


FIG. 6.—Sequencing analysis of arthrin peptides from *D. melanogaster* and *L. griseus*. (*a*) HPLC MS zoom scan and HPLC MS/MS at 1806.4 Da of the tryptic digest of arthrin from *D. melanogaster*. (*b*) HPLC MS zoom scan and HPLC MS/MS at 1813.4 Da of the tryptic digest of the arthrin and actin mixture from *L. griseus*.

whereas in the Heteroptera, four wings are normally present, but with most flapping from the hind wings (Grodnitsky 1995). Neither does arthrin appear to be strongly related to body size (and hence wing-beat frequency): for example, arthrin is found in the very small Chironomidae, as well as the giant water bug *Lethocerus*. Nor are there any obvious commonalities of natural history: arthrin is found in both aquatic and terrestrial taxa, whether phytophagous, sarcophagous, predatory, haemophagous, or detritivorous. Given these observations,

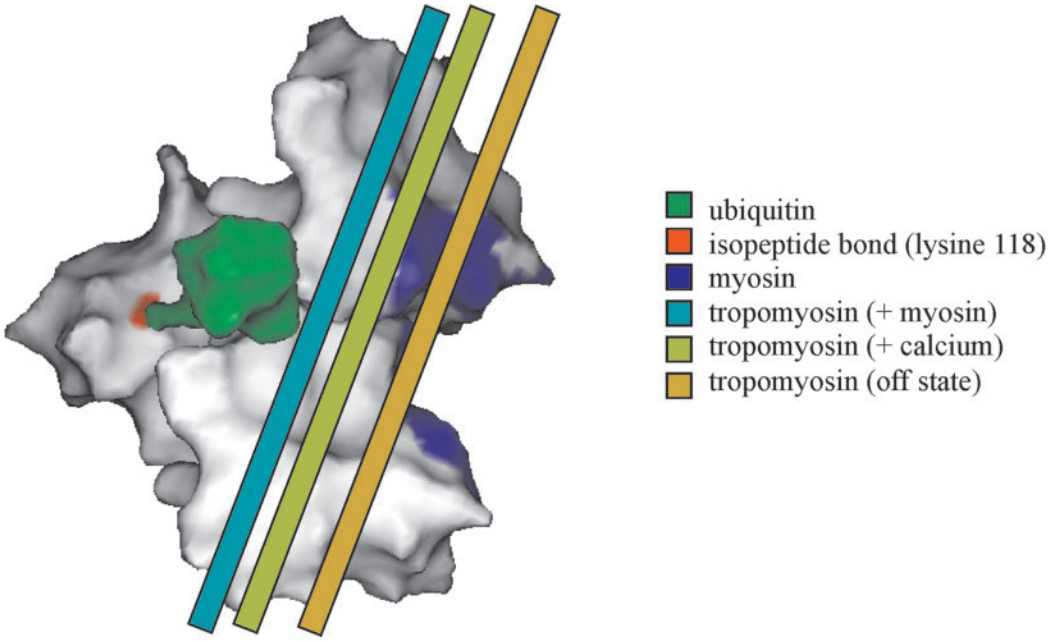


FIG. 7.—Model of ubiquitin docked onto the site of the isopeptide bond and molecular footprints of myosin and tropomyosin on F-actin. Ubiquitin was positioned towards the location of tropomyosin in the presence of myosin under the restraints of a nonbonded clash function (for details see *Materials and Methods*). The myosin footprint shows the binding sites of a single myosin head binding to the two actin monomers on the right as identified by the studies of Rayment et al. (1993). The tropomyosin positions are based on studies of skeletal muscle thin filaments by Vibert, Craig, and Lehman (1997). Actin orientation and tropomyosin representation were chosen to resemble figure 2 in McGough (1998).

we speculate that arthrin has simply not arisen in most insect taxa. Where it has arisen it has possibly been strongly selected by some improvement in asynchronous muscle function and become highly conserved evolutionarily. Such a scenario is consistent with the convergent but conserved evolution of other muscle proteins, such as the actins (Mounier and Sparrow 1993), and of asynchronous muscle itself (Pringle 1981).

The site of the isopeptide bond in arthrin, between lysine 118 of the actin and the C-terminal glycine 76 of ubiquitin, is the same for two species from evolutionary distant orders, *D. melanogaster* and *L. griseus*. Parsimony analysis suggests that arthrin has evolved independently in each of these orders but with extreme convergence in that the ubiquitination sites are identical. This specificity suggests some functional relevance. In the "conventional" view of the structure of actin (Kabsch et al. 1990), lysine 118 lies on the surface of the molecule on the backside of subdomain 1. Lysine 118 is part of the α-helix 112 to 125. In the F-actin model (Lorenz, Popp, and Holmes 1993), the side chain of lysine 118 cannot form any intramolecular or intermolecular bonds. The F-actin model also shows that the self-association sites of F-actin are all distant from lysine 118. This is consistent with the in vitro ability of arthrin to polymerize (Bullard et al. 1985; Schmitz et al. 1998; Burgess et al. 2000; Galkin et al. 2003). The site of the isopeptide bond is in agreement with the three-dimensional reconstructions of electron micrographs of arthrin, which suggest ubiquitin is located in the same region (Burgess et al. 2000; Galkin et al. 2003). The lysine 118 side chain is highly accessible to the outside environment. Actin subdomains 1 and 2, the so-called outer domain, are generally favorite targets for F-actin binding proteins, and its high accessibility to actin-binding proteins may be the reason for the frequency of these interactions (McGough 1998). Actin residues 86 to 117 have been implicated in binding the Z-disc protein α-actinin (e.g., Lebart et al. 1993) and cryoelectron microscopy and image analysis confirmed this region as one of the two contact sites for α-actinin (McGough, Way, and DeRosier 1994). Lysine 118 is either part of or directly next to this contact site. While the distribution of arthrin within the sarcomere remains unclear, it is also not clear if proximity of the isopeptide bond in arthrin and the α-actinin contact site has any functional relevance. Myosin binds to regions on subdomain 1, 2, and 3 of actin (Rayment et al. 1993; Schröder et al. 1993), but lysine 118 is neither part of nor near these myosin-binding sites, consistent with in vitro results showing that the interactions of arthrin with myosin were identical to those of actin (Bullard et al. 1985; Schmitz et al. 1998).

In the absence of Ca^{2+}, troponin makes contacts with the extreme periphery of subdomain 1 and ends over subdomain 2 of the same actin monomer (Lehman et al. 2001). Actin residues 1 to 4, 23 to 27, and 47 are thought to be involved in troponin-I binding (Perry 1999; Luo et al. 2000). With regard to the site of the isopeptide bond and the size of ubiquiuitin, it appears unlikely that troponin and the ubiquitin of arthrin have overlapping binding sites on actin. Nevertheless, in their recent structural study on arthrin, Galkin et al. (2003) pointed out that in the "on" state, the unusually large troponin complex of insect flight muscles (Bullard et al. 1988) might make, at rest length, an unfavorable contact with the ubiquitin attached to the opposite actin strand, making stretch required to fully activate the muscle. Possible artifacts from negative

staining and their use of in vitro polymerized F-arthrin in which, unlike in vivo, every actin subunit is arthrin, have to be considered. The authors observation of disorder in the position of the ubiquitin also means that the position in the reconstruction can only be seen as an average one. Our model shown in figure 7 is the result of a rather different approach. We make use of having unambiguously identified the site of the isopeptide in arthrin by docking ubiquitin on the actin filament, tethered by its flexible tail region to lysine 118 on actin. Considering bulk steric consequences with a simple nonclash approach, we then determine whether the regulatory proteins on actin can be reached by the ubiquitin. Nevertheless, interpretation of our results is along similar lines, but we speculate on arthrin possibly stabilizing tropomyosin in the "on" state: In the low-Ca^{2+} state, tropomyosin lies over the outer domain of actin, blocking strong myosin binding (Lehman, Craig, and Vibert 1994). Addition of Ca^{2+} causes an azimuthal movement of tropomyosin about 25° towards the inner domain of actin, still covering some of the myosin-binding interface. Myosin binding in rigor causes a further shift of tropomyosin about 10°, such that it now lies entirely over the inner domain of actin, leaving all of the myosin-binding sites accessible (Vibert, Craig, and Lehman 1997). In arthrin, this further shift would bring the tropomyosin well within the potential range of the ubiquitin bound at lysine 118 at the rear surface of the monomer of the other strand of the two-start actin helix (fig. 7). This observation allows us to speculate on a functional role for arthrin.

Several lines of evidence suggest that electrostatic charges on the actin surface play critical roles in determining the equilibrium between states of actin-tropomyosin that is a central feature of the steric-blocking mechanism of actin filament regulation (Vibert, Craig, and Lehman 1997). Bing et al. (1998), for example, propose that a cluster of negative charges on subdomain 2 of actin promotes the "on" state by repulsion of the negatively charged tropomyosin. In the absence of Ca^{2+}, binding of the positively charged troponin-I to these negative charges on actin might then neutralize the repulsion and thereby causing tropomyosin to switch to the "off" state. This is also compatible with the general observation that inhibitory proteins such as troponin-I and caldesmon bind to actin rather than to tropomyosin (Mornet et al. 1995). In the "on" state, the negatively charged tropomyosin lies in a trough of positive charges of the actin (Lehman et al. 1995; Vibert, Craig, and Lehman 1997). Since it is proposed that tropomyosin is stabilized in the "on" state by actin surface charges, it seems reasonable to propose that the role of ubiquitin in arthrin might very well be to further stabilize the "on" state. The theoretical isoelectric points of tropomyosin, actin, ubiquitin, and troponin-I appear to support this idea. With a pI of 4.69, tropomyosin (P09493) is clearly the most acidic one, followed by actin (P02575) with a pI of 5.29. Ubiquitin (P02248) and troponin-I (P48788) are much more basic with pIs of 6.56 and 8.88, respectively. The idea of arthrin being involved in thin filament regulation could now be tested by structural and solution studies. The role of arthrin could be a static or a regulated one. Possible advantages of stabilizing tropomyosin in the "on" state for the function of asynchronous flight muscle have to be discussed.

Further, knowledge of the site of the isopeptide bond has allowed us to investigate the function of the protein in vivo. By mutating the respective lysine forming the isopeptide bond we made a *D. melanogaster* line in which the actin of the indirect flight muscle does not become ubiquitinated (Schmitz et al., unpublished data). This confirms that lysine 118 is the unique site of the isopeptide bond. Since in the absence of arthrin, flies were able to fly, arthrin cannot be essential for flight in *D. melanogaster*, but current investigations suggest that there are significant quantitative changes in the flight ability. We hope that studies of these mutant flies will finally elucidate the role of arthrin.

Acknowledgments

We thank Terry Crawford, Alistair Darby, and Geoff Oxford for providing specimens, Chris Rees for help with identification, and BBSRC, the Royal Society and the German National Merit Foundation, for grant support.

Literature Cited

Ashman, K., T. Houthaeve, J. Clayton, M. Wilm, A. Podtelejnikov, O. N. Jensen, and M. Mann. 1997. The application of robotics and mass spectrometry to the characterisation of the *Drosophila melanogaster* indirect flight muscle proteome. Lett. Pept. Sci. **4**:57–65.

Ball, E., C. C. Karlik, C. J. Beall, D. L. Saville, J. C. Sparrow, B. Bullard, and E. A. Fyrberg. 1987. Arthrin, a myofibrillar protein of insect flight muscle, is an actin ubiquitin conjugate. Cell **51**:221–228.

Benton, M. J. 1993. The fossil record 2. Chapman and Hall, London.

Bing, W., A. Razzaq, J. C. Sparrow, and S. Marston. 1998. Tropomyosin and troponin regulation of wild-type and E93K mutant actin filaments from *Drosophila* flight muscle—charge reversal on actin changes actin-tropomyosin from on to off state. J. Biol. Chem. **273**:15016–15021.

Brandimarti, R., and B. Roizman. 1997. Us9, a stable lysineless herpes simplex virus 1 protein, is ubiquitinated before packaging into virions and associates with proteasomes. Proc. Natl. Acad. Sci. USA **94**:13973–13978.

Bullard, B., J. Bell, R. Craig, and K. Leonard. 1985. Arthrin: a new actin-like protein in insect flight muscle. J. Mol. Biol. **182**:443–454.

Bullard, B., K. Leonard, A. Larkins, G. Butcher, C. Karlik, and E. Fyrberg. 1988. Troponin of asynchronous flight muscle. J. Mol. Biol. **204**:621–637.

Burgess, S. A., P. J. Knight, M. L. Walker, S. Schmitz, J. C. Sparrow, and J. Trinick. 2000. Real-space 3-D reconstruction of frozen-hydrated arthrin and actin filaments at 2 nm resolution. Biophys. J. **78**:47(A).

Carver, M., G. F. Gross, and T. E. Woodward. 1991. Hemiptera. Pp. 429–509 *in* I. D. Naumann et al., eds. CSIRO, The insects of Australia. Cornell University Press, Ithaca, New York.

Caves, L. S. D., M. J. Hartshorn, S. J. P. Mumford, M. Afshar, J. E. Molloy, and R. E. Hubbard. 1997. An interactive modelling system for the mechanical manipulation of macromolecules and supramolecular assemblies. J. Muscle Res. Cell Motil. **118**:186–187(A)

Chinery, M. 1986. Collins guide to the insects of Britain and Western Europe. Collins, London.

Cullen, M. J. 1974. The distribution of asynchronous muscle in insects with particular reference to Hemiptera: an electron microscope study. J. Entomol. A. **49**:17–41.

Dickinson, M. H., and M. S. Tu. 1997. The function of dipteran flight muscle. Comp. Biochem. Physiol. **116**:223–238.

Farris, J. S. 1969. Methods for computing Wagner trees. Syst. Zool. **19**:82–92.

———. 1989. The retention index and the rescaled consistency index. Cladistics **5**:417–419.

Field, S. J., J. C. Pinder, B. Clough, A. R. Dluzewski, R. J. Wilson, and W. B. Gratzer. 1993. Actin in the merozoite of the malaria parasite, *Plasmodium falciparum*. Cell Motil. Cytoskel. **25**:43–48.

Fitch, W. M. 1971. Towards defining the course of evolution: minimal change for a specific tree topology. Syst. Zool. **20**: 406–416.

Friedrich, M., and D. Tautz. 1997. Evolution and phylogeny of the Diptera: a molecular phylogenetic analysis using 28s rDNA sequences. Syst. Biol. **46**:674–698.

Galkin, V. E., A. Orlova, N. Lukoyanova, M. S. VanLoock, P. Haag, B. Bullard, and E. H. Egelman. 2003. The location of ubiquitin in Lethocerus arthrin. J. Mol. Biol. **325**: 623–628.

Gauld, I., and B. Bolton. 1988. The Hymenoptera. Oxford University Press, Oxford.

Grodnitsky, D. L. 1995. Evolution and classification of insect flight kinematics. Evolution **49**:1158–1162.

Hennig, W. 1981. Insect phylogeny. John Wiley and Sons, Chichester, U.K.

Hershko, A., E. Leshinsky, D. Ganoth, and H. Heller. 1984. ATP-dependent degradation of ubiquitin-protein conjugates. Proc. Natl. Acad. Sci. USA **81**:1619–1623.

Hoaglund-Hyzer, C. S., and D. E. Clemmer. 2001. Ion trap/ion mobility/quadrupole/time of flight mass spectrometry for peptide mixture analysis. Anal. Chem. **73**:177–184.

Iwaya, K., H. Nishibori, T. Osada et al. (12 co-authors). 1997. Immunoreaction at 43 kDa with anti-ubiquitin antibody in breast neoplasms. Jpn. J. Cancer Res. **88**:273–280.

Kabsch, W., H. G. Mannherz, D. Suck, E. F. Pai, and K. C. Holmes. 1990. Atomic structure of the actin:DNase I complex. Nature **347**:37–44.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**: 680–685.

Laub, M., J. A. Steppuhn, M. Bluggel, D. Immler, H. E. Meyer, and H. P. Jennissen. 1998. Modulation of calmodulin function by ubiquitin-calmodulin ligase and identification of the responsible ubiquitylation site in vertebrate calmodulin. Euro. J. Biochem. **255**:422–431.

Lebart, M.-C., C. Mejean, C. Roustan, and Y. Benyamin. 1993. Further characterization of the alpha-actinin-actin interface and comparison with filamin-binding sites on actin. J. Biol. Chem. **268**:5642–5648.

Lehman, W., R. Craig, and P. Vibert. 1994. Ca^{2+}-induced tropomyosin movement in Limulus thin-filaments revealed by 3-dimensional reconstruction. Nature **368**:65–67.

Lehman, W., M. Rosol, L. S. Tobacman, and R. Craig. 2001. Troponin organization on relaxed and activated thin filaments revealed by electron microscopy and three-dimensional reconstruction. J. Mol. Biol. **307**:739–744.

Lehman, W., P. Vibert, P. Uman, and R. Craig. 1995. Steric-blocking by tropomyosin visualized in relaxed vertebrate muscle thin filaments. J. Mol. Biol. **251**:191–196.

Lorenz, M., D. Popp, and K. C. Holmes. 1993. Refinement of the F-actin model against x-ray fiber diffraction data by the use of a directed mutation algorithm. J. Mol. Biol. **234**: 826–836.

Luo, Y., J. Leszyk, B. Li, Z. X. Li, J. Gergely, and T. Tao. 2000. Residues in the inhibitory and the second actin-binding regions of skeletal muscle troponin-I photocrosslink to different actin subunits in the reconstituted thin filament. Biophys. J. **78**:2316(A).

Maddison, W. P., and D. R. Maddison. 1992. MacClade: analysis of phylogeny and character evolution. Version 3. Sinauer Associates, Sunderland, Mass.

Mayer, A., N. R. Siegel, A. L. Schwartz, and A. Ciechanover. 1989. Degradation of proteins with acetylated amino termini by the ubiquitin system. Science **244**:1480–1483.

McAlpine, J. F. 1989. Phylogeny and classification of the Muscomorpha. Pp. 1397–1518 *in* J. F. McAlpine and D. M. Wood, eds. Manual of nearctic Diptera, Vol. 3. Ottawa, Canada.

McGough, A. 1998. F-actin-binding proteins. Curr. Opin. Struct. Biol. **8**:166–176.

McGough, A., M. Way, and D. DeRosier. 1994. Determination of the alpha-actinin binding site on actin filaments by cryo-electron microscopy and image analysis. J. Cell Biol. **126**: 433–443

Mornet, D., A. Bonetkerrache, G. M. Strasburg, V. B. Patchell, S. V. Perry, P. A. J. Huber, S. B. Marston, D. A. Slatter, J. S. Evans, and B. A. Levine. 1995. The binding of distinct segments of actin to multiple sites in the C-terminus of caldesmon—comparative aspects of actin interaction with troponin-I and caldesmon. Biochem. **34**:1893–1901.

Mounier, N., and J. C. Sparrow. 1993. Muscle actin genes in insects. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **105**:231–238.

Nickel, B. E., C. D. Allis, and J. R. Davie. 1989. Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. Biochemistry **28**:958–963.

Oldroyd, H. 1970. Diptera 1. Introduction and key to families. Handbook for the Identification of British Insects. 3rd Edition. Royal Entomological Society of London, London.

Peckham, M., R. Cripps, D. White, and B. Bullard. 1992. Mechanics and protein content of insect flight muscles. J. Exp. Biol. **168**:57–76.

Perry, S. V. 1999. Troponin I: Inhibitor or facilitator. Mol. Cell. Biochem. **190**:9–32.

Pringle, J. W. S. 1981. The evolution of fibrillar muscle in insects. J. Exp. Biol. **94**:1–14.

Rayment, I., W. R. Rypniewski, K. Schmidtbase, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden. 1993. 3-Dimensional structure of myosin subfragment-1—a molecular motor. Science **261**: 50–58.

Razzaq, A., S. Schmitz, C. Veigel, J. E. Molloy, M. A. Geeves, and J. C. Sparrow. 1999. Actin residue E93 is identified as an amino acid affecting myosin binding. J. Biol. Chem. **274**:28321–28328.

Schmitz, S., J. Clayton, U. Nongthomba, C. Veigel, M. A. Geeves, and J. C. Sparrow. 2000. *Drosophila* Act88F indirect flight muscle-specific actin is not N-terminally acetylated: a mutation in N-terminal processing affects actin function. J. Mol. Biol. **295**:201–210.

Schmitz, S., A. Razzaq, M. A. Geeves, and J. C. Sparrow. 1998. Kinetic characterization of the *Drosophila melanogaster* indirect flight muscle-specific actins ACT88F, the ACT88F mutant E93K and the ubiquitinated ACT88F arthrin using the stopflow. J. Muscle Res. Cell Motil. **19**:300(A).

Schröder, R. R., D. J. Manstein, W. Jahn, H. Holden, I. Rayment, K. C. Holmes, and J. A. Spudich. 1993. 3-Dimensional atomic model of F-actin decorated with Dictyostelium myosin S1. Nature **364**:171–174.

Schuh, R. T. 1979. Review of Cobben: evolutionary trends in Heteroptera. Syst. Zool. **28**:653–656.

Schuh, R. T., and J. A. Slater. 1995. True bugs of the world (Hemiptera: Heteroptera). Classification and natural history. Cornell University Press, Ithaca, New York.

Schuh, R. T., and P. Stys. 1991. Phylogenetic analysis of cimicomorphan family relationships (Heteroptera). J. N. Y. Entomol. Soc. **3**:298–350.

Sheterline, P., J. Clayton, and J. C. Sparrow. 1998. Actin: protein profile. 4th edition. Oxford University Press, Oxford.

Skinner, B. 1998. The colour identification guide to moths of the British Isles. 2nd edition. Viking, Harmondsworth, Middlesex, England.

Sous, C., U. Pelikan, P. Püntmann, and G. Beinbrech. 1984. Arthrin content of myofibrils and actin filaments of various insect muscles. J. Muscle Res. Cell Motil. **5**:204(A).

Sun, Z.-W., and C. D. Allis. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature **418**:104–108.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24**: 4876–4882.

Vibert, P., R. Craig, and W. Lehman. 1997. Steric-model for activation of muscle thin filaments. J. Mol. Biol. **266**: 8–14.

Weber, P. C., S. J. Spatz, and E. C. Nordby. 1999. Stable ubiquitination of the ICPOR protein of herpes simplex virus type 1 during productive infection. Virology **253**: 288–298.

Wheeler, W. C., R. T. Schuh, and R. Bang. 1993. Cladistic relationships among higher groups of Heteroptera: congruence between morphological and molecular data sets. Ent. Scand. **24**:121–137.

Wheeler, W. C., M. Whiting, Q. D. Wheeler, and J. M. Carpenter. 2001. The phylogeny of the extant hexapod orders. Cladistics **17**:113–169.

White, D. C. S. 1983. The elasticity of relaxed insect fibrillar flight muscle. J. Physiol. **343**:31–57.

Wiegmann, B. M., C. Mitter, and F. C. Thompson. 1993. Evolutionary origin of the Cyclorrhapha (Diptera): tests of alternative morphological hypotheses. Cladistics **9**:41–81.

Woodley, N. E. 1989. Phylogeny and classification of the ''orthorrhaphous'' Brachycera. Pp. 371–1395 *in* J. F. McAlpine and D. M. Woods, eds. Manual of nearctic Diptera, Vol 3. Agriculture Canada, Ottawa, Canada.

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Accepted July 7, 2003