Insect Biochemistry and Molecular Biology 62 (2015) 174–182

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Myofilament proteins in the synchronous flight muscles of *Manduca sexta* show both similarities and differences to *Drosophila melanogaster*

Agnes Ayme-Southgate*, Samuel Feldman, Diana Fulmer

Department of Biology, College of Charleston, Charleston, SC, USA

ARTICLE INFO

Article history:
Received 4 October 2014
Received in revised form
26 January 2015
Accepted 17 February 2015
Available online 19 March 2015

Keywords:
Flight muscles
Synchronous
Troponin
Stretch activation
Thick-thin filament

ABSTRACT

Insect flight muscles have been classified as either synchronous or asynchronous based on the coupling between excitation and contraction. In the moth *Manduca sexta*, the flight muscles are synchronous and do not display stretch activation, which is a property of asynchronous muscles. We annotated the *M. sexta* genes encoding the major myofibrillar proteins and analyzed their isoform pattern and expression. Comparison with the homologous genes in *Drosophila melanogaster* indicates both difference and similarities. For proteins such as myosin heavy chain, tropomyosin, and troponin I the availability and number of potential variants generated by alternative spicing is mostly conserved between the two insects. The exon usage associated with flight muscles indicates that some exon sets are similarly used in the two insects, whereas others diverge. For actin the number of individual genes is different and there is no evidence for a flight muscle specific isoform. In contrast for troponin C, the number of genes is similar, as well as the isoform composition in flight muscles despite the different calcium regulation. Both troponin I and tropomyosin can include COOH-terminal hydrophobic extensions similar to tropomyosinH and troponinH found in *D. melanogaster* and the honeybee respectively.

1. Introduction

The structure of the muscle contractile unit (sarcomere) is highly conserved throughout evolution, together with a parallel significant conservation of the main myofibrillar proteins. In insects the mechanical demands associated with flight have generated a variety of flight muscle types with varied anatomical and physiological adaptations. One of the main physiological differences between insect flight muscles, which are all striated, resides with the coupling between nerve excitation and muscle contraction. Most commonly, one nerve excitation leads to one muscle contraction, a physiological mode known as synchronous that is also representative of all vertebrate striated voluntary and cardiac muscles (Dudley, 1991). In all adult insects, synchronous muscles are involved in all non-flight activities such as walking and feeding. In basal insects (e.g. dragonflies and locusts), flight muscles are also synchronous (Dudley, 1991). Several orders of insects have developed an alternative physiological system known as the asynchronous mode where there is a neuro-muscular decoupling, with many contractions for each initial nerve impulse (Pringle, 1978; Josephson et al., 2000). How this is accomplished is still largely unexplained, but is known to depend on a process described as "delayed increase in tension following stretch" or "stretch activation" (Pringle, 1978). While there are no "real" asynchronous muscles in vertebrate systems, cardiac muscles of many species show some of the characteristics of asynchronous physiology, in particular an enhanced tension in response to stretch (the Frank-Starling mechanism, reviewed in Shiels and White, 2008).

Lepidoptera occupy a special phylogenetic place as they are holometabolous (undergoing complete metamorphosis), share many derived traits with insects such as Diptera and Hymenoptera, yet display synchronous flight muscles (Pringle, 1978, Dudley, 2000; Syme and Josephson, 2002). Lepidoptera together with several other insect orders support the hypothesis of multiple acquisitions and/or losses of asynchronous flight during insect evolutionary history (Pringle, 1981). As shown in Fig. 1 there are several ways to explain the phylogenetic distribution of

Abbreviations: IFM, Indirect Flight Muscle.

* Corresponding author. Department of Biology, College of Charleston, 66 George Street, Charleston, SC 29401, USA. Tel.: +843 953 6544; fax: +843 953 5453.
E-mail address: southgatea@cofc.edu (A. Ayme-Southgate).

http://dx.doi.org/10.1016/j.ibmb.2015.02.008
0965-1748/

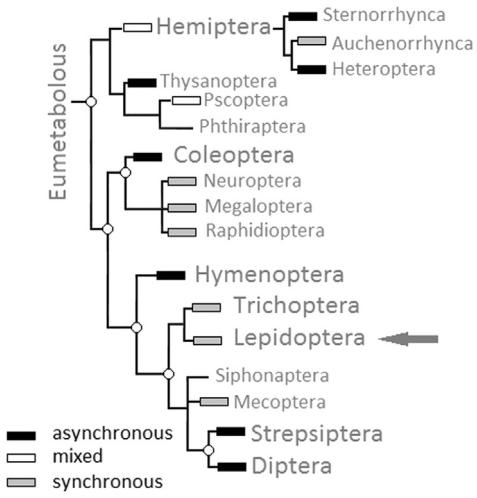

Fig. 1. Phylogenetic tree of the major holometabolous insect orders indicating the distribution of asynchronous versus synchronous flight muscles. This phylogeny supports the hypothesis of multiple acquisition and/or loss for the asynchronous muscle physiology.

synchronous and asynchronous physiologies within the holometabolous insects. Either asynchrony was already acquired in more basal insects and lost at several points including in the common ancestor of Trichoptera and Lepidoptera or synchrony was still the basal state and was acquired independently in Hemiptera, Coleoptera, Hymenoptera and the common ancestor of Strepsidera and Diptera. A combination of multiple acquisitions and losses has been proposed (Dudley, 2000).

One constant denominator from studies of myofilament proteins in *Drosophila melanogaster*, *Apis mellifera*, and *Lethocerus indicus* is that most proteins of the myofibrillar filaments (actin, myosin, etc.) are represented by several isoforms, generated by two basic mechanisms, either (1) the presence of gene families where variants of one protein are encoded by different genes (e.g. troponin C) or (2) the alternative splicing of a primary mRNA from a unique gene (e.g. myosin heavy chain) (reviewed in Bernstein et al., 1993; Maughan and Vigoreaux, 1999; Hooper and Thuma, 2005; Bullard and Pastore, 2011). In these three insects many myofibrillar protein genes express one flight muscle-specific isoform. Studies in *D. melanogaster* also tested the importance of these protein variants for the assembly and stability of the asynchronous indirect flight muscle (IFM) sarcomeres, as well as flight performance (e.g. Miller et al., 2007, 2009; Swank et al., 2004, 2006; Suggs et al., 2007; Yang et al., 2008). Because these three insect species use asynchronous flight muscles and based on the genetic analyses performed in *D. melanogaster*, the implication has been that the asynchronous-specific isoforms are essential adaptations to asynchronous flight. However, some of these adaptations are present in some but not all insects with asynchronous muscles, as it is the case for the proteins arthrin (a modified form of actin), which is found in Hemiptera, but not in Hymenoptera (Schmitz et al., 2003) and flightin (Vigoreaux et al., 1993), which in *Drosophila*, is asynchronous muscle-specific, but is found in synchronous flight muscles of many other insects, and even other arthropods (Soto-Adames et al., 2014).

We annotated the genes for the major myofibrillar protein found in the *Manduca sexta* genome and analyzed the possibility of alternative splicing pattern. The expression pattern was determined for a number of myofibrillar proteins in both flight and non-flight muscles of *M. sexta*. We discussed the differences and similarity between the variant sets expressed in *M. sexta* synchronous flight muscles and *D. melanogaster* asynchronous IFMs and the implications for synchronous physiology.

2. Materials and methods

2.1. Insects and RNA sample preparation

Manduca sexta were purchased as larvae and/or pupae from Educational Science (TX) and reared up to emergence of the imago at which point they were sacrificed. Total RNA was purified from whole animals, isolated body parts (legs, heads, thoraces), and from dissected flight muscles using Trizol followed by PureLink purification kits (Invitrogen™) as previously described (Ayme-Southgate et al., 2008).

2.2. Bioinformatics analysis

D. melanogaster protein sequences for the various protein isoforms were retrieved from GenBank. The corresponding contigs were isolated from the *M. sexta* genome database using tblastn searches. Alignments with available RNAseq data, as well as *in silico* predictions of exon-intron structure were visualized in the Apollo browser. Incorrect splice sites, missing or extraneous exons were manually annotated by examination of the open reading frame and comparison with the corresponding *Drosophila* sequences. Sequence comparisons were carried out using the CLUSTALW2 algorithm, and the alignments were viewed in Jalview (Thompson et al., 1994, 1997).

2.3. Expression analysis

RT-PCR reactions were performed, as described before, with different RNA preparations and primer sets (Southgate and Ayme-Southgate, 2001). Annealing for both the RT and PCR reactions were tested using a range of temperatures to optimize each primer set. If the splice sites or new exon needed to be confirmed, the DNA fragments were isolated after agarose gel electrophoresis followed by sequencing (Genewiz Inc.).

3. Results

3.1. Myosin heavy chain (MHC)

A unique gene with complex alternative splicing is responsible for generating all the myosin heavy chain isoforms in *M. sexta*. The *Manduca* gene is composed of 25 exons sets compared to 19 in *D. melanogaster* with seven sets of mutually-exclusive exons where only one of two or more exons is retained in mRNAs after splicing (Fig. 2A). Other gene characteristics are summarized in the Supplementary Table.

As shown in Table 1, each mutually exclusive exon set in the MHC gene of *M. sexta (Ms)* codes for the same region of the protein as in *D. melanogaster (Dm)*. On the other hand the third and fourth exon sets contain different numbers of variants between *Ms* and *Dm* (Table 1). There is one extra alternative exon set (#14) with two mutually exclusive variants in the *M. sexta* MHC gene compared to *D. melanogaster*. Exons 12 to 16 in the *M. sexta* MHC gene correspond to one large exon (#10) in the *Drosophila* gene, and therefore the possibility of alternative splicing does not exist for that region in *D. melanogaster*. This additional alternative exon set has been also described in other insects including several Lepidoptera (Kollmar and Hatje, 2014). Therefore in the two insects the MHC isoforms vary mainly within the same regions of the protein, but the number of possible variants is different. The linear order of the alternative exons in each exon set does not imply sequence