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Commentary

The long and winding road: influences of intracellular metabolite diffusion on cellular organization and metabolism in skeletal muscle

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Summary

A fundamental principle of physiology is that cells are small in order to minimize diffusion distances for O_2 and intracellular metabolites. In skeletal muscle, it has long been recognized that aerobic fibers that are used for steady state locomotion tend to be smaller than anaerobic fibers that are used for burst movements. This tendency reflects the interaction between diffusion distances and aerobic ATP turnover rates, since maximal intracellular diffusion distances are ultimately limited by fiber size. The effect of diffusion distance on O_2 flux in muscle has been the subject of quantitative analyses for a century, but the influence of ATP diffusion from mitochondria to cellular ATPases on aerobic metabolism has received much less attention. The application of reaction–diffusion mathematical models to experimental measurements of aerobic metabolic processes has revealed that the extreme diffusion distances between mitochondria found in some muscle fibers do not necessarily limit the rates of aerobic processes *per se*, as long as the metabolic process is sufficiently slow. However, skeletal muscle fibers from a variety of animals appear to have intracellular diffusion distances and/or fiber sizes that put them on the brink of diffusion limitation. Thus, intracellular metabolite diffusion likely influences the evolution of muscle design and places limits on muscle function.

Key words: muscle fiber, fiber growth, diffusion, metabolic modeling, reaction-diffusion, exercise, metabolism, scaling, crustacean, fish, phosphagen, arginine phosphate, arginine kinase, creatine phosphate, creatine kinase, mitochondria.

Introduction

Intracellular reaction rates are dependent both on the catalytic properties of enzymes and on the diffusive transport of substrates to enzyme active sites. However, most metabolic studies have focused only on reaction kinetics, neglecting potential effects of diffusion. This simplified approach has arisen because cells are generally considered to be small, meaning that maximal intracellular diffusion distances are short and diffusion of metabolites is therefore very fast relative to the rate of metabolic processes. In mammalian skeletal muscle, this view is supported by the close matching of purely kinetic models to experimental data for some of the major pathways of energy metabolism (e.g. Vicini and Kushmerick, 2000; Lambeth and Kushmerick, 2002; Korzeniewski, 2003). However, diffusion will exert greater influence over cellular metabolism as either the intracellular diffusion distances increase or the rate of metabolic processes increase (Weisz, 1973).

The maximal aerobic metabolic rate in muscle is largely dependent on the mitochondrial content. For example, very low rates of aerobic metabolism are observed in some fish white muscles, which may devote <1% of fiber volume to mitochondria, whereas extremely high metabolic rates are observed in insect flight muscle, where dense clusters of mitochondria may exceed 40% of the fiber volume. Thus, the diversity of muscle structure and function found in nature encompasses an aerobic ATP demand that can range from <0.1 to >2000 μmol g^{-1} min^{-1}, and intracellular diffusion distances between mitochondria that range from <1 to several hundred μm. Quantitative analyses of some skeletal muscle types indicate that the extent to which metabolite diffusion limits aerobic flux is variable (e.g. Mainwood and Rakusan, 1982; Meyer et al., 1984; Tyler and Sidell, 1984; Egginton and Sidell, 1989; Hubley et al., 1997; Kemp et al., 1998; Kinsey et al., 2005; Hardy et al., 2006; Nyack et al., 2007), but a broad analysis that encompasses diffusion effects over the full spectrum of muscle fiber designs is lacking. Thus, the extent to which intracellular transport processes govern rates of energy metabolism and influence the evolution of muscle metabolic structure remains unresolved.

Energy transport in muscle fibers

Aerobic metabolic processes rely on the transport of ATP from mitochondria to sites of utilization in the cytoplasm, and the spacing between mitochondria dictates the distance over

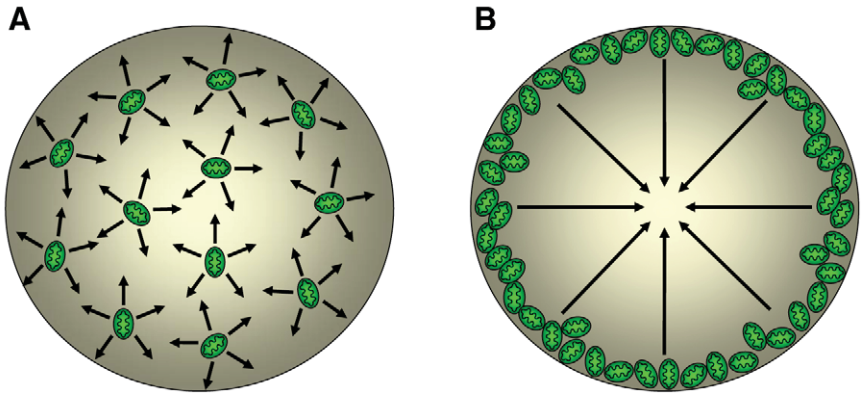

Fig. 1. A schematic of a muscle fiber in cross section shows the influence of mitochondrial distribution on diffusion distance. While mitochondrial distribution can be complex, shown here are two idealized patterns where mitochondria may be distributed throughout the fiber, leading to short diffusion distances (A; intermyofibrillar mitochondria), or clustered exclusively at the sarcolemmal membrane, leading to longer diffusion distances (B; subsarcolemmal mitochondria). More commonly, a combination of both subsarcolemmal and intermyofibrillar mitochondria are observed. ATP must be transported *via* diffusion (black arrows) from sites of production in the mitochondria (colored green) to cellular ATPases throughout the cytoplasm. Likewise, ADP must be transported back to the mitochondria (not shown). In some fibers the mitochondrial distribution changes during fiber growth from primarily intermyofibrillar to primarily subsarcolemmal, meaning the diffusion distance between mitochondria may change dramatically as muscle fibers increase in size (see text).

which diffusion must occur. Mitochondria are generally classified into one of two types: those distributed throughout the muscle fiber are called intermyofibrillar mitochondria, and those clustered at the periphery of the fiber adjacent to the sarcolemmal membrane are called subsarcolemmal mitochondria (Fig. 1). While most fibers have both kinds of mitochondria in various proportions, it is clear from Fig. 1 that the arrangement and density of mitochondria can greatly affect the diffusion distance for ATP.

In skeletal muscle and other tissues, the utilization of ATP produced by mitochondria is mediated by phosphagen kinases, such as creatine kinase (CK) and arginine kinase (AK). Phosphagen kinases are found in vertebrates and invertebrates, and are prevalent in tissues such as muscle that have high or variable rates of ATP demand (Ellington, 2001). These enzymes catalyze the reversible transfer of a phosphoryl group from a phosphagen, which for CK is phosphocreatine (PCr) and for AK is arginine phosphate (AP), to ADP, forming ATP:

$$\text{PCr/AP} + \text{ADP} + \text{H}^+ \leftrightarrow \text{Creatine/Arginine} + \text{ATP} .$$

Thus, a primary function of phosphagen kinases is to preserve ATP concentration (at the expense of PCr/AP concentration) during fluctuations in ATP demand, such as during rest to work transitions. This role of phosphagen kinases is known as temporal buffering of ATP concentration.

In addition, PCr and AP facilitate transport of ATP from mitochondria to sites of demand by virtue of the fact that both of these molecules freely diffuse, offering a parallel pathway of high-energy phosphate diffusive flux (Meyer et al., 1984; Ellington, 2001). That is, since the high-energy phosphate on PCr or AP is rapidly exchanged with that on ATP, these phosphagens can be thought of as carriers of high energy phosphates that supplement the direct diffusion of ATP (Fig. 2). In fact, since PCr and AP occur in higher concentrations and have higher diffusion coefficients than ATP, the vast majority of high-energy phosphate transport from mitochondria to cellular ATPases in muscle occurs by the diffusion of phosphagen, rather than directly as ATP (Fig. 2) (Meyer et al., 1984; Ellington and Kinsey, 1998).

The view of energy transport in muscle described above may not fully characterize all systems. For instance, there are multiple isoenzymes of phosphagen kinases that are localized to different subcellular regions, and more complex ATP delivery pathways have been proposed (reviewed in Ellington, 2001). However, a full discussion of phosphagen kinase function is beyond the scope of this paper, and our purpose is to focus more generally on the rules of cellular energy transport as they apply to both traditional model systems, for which there is a wealth of detailed metabolic data, and non-traditional model systems, for which there is not.

Metabolite diffusion in muscle

The cytoplasm is a complex and crowded medium consisting of soluble and bound macromolecules, fibrous elements and an array of membrane-bound organelles (reviewed in Luby-Phelps, 2000). In skeletal muscle from a diversity of organisms, the diffusion coefficient (D) of phosphagens, ATP and other metabolites is both time-dependent and anisotropic, meaning

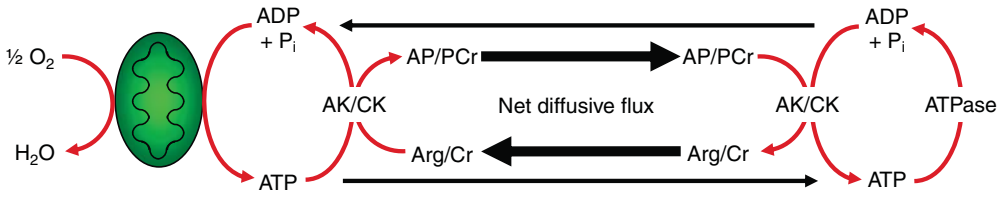

Fig. 2. Phosphagen kinases (AK or CK) mediate most diffusive flux of ATP in muscle. The red arrows indicate enzymatic catalysis and the black arrows net diffusive flux. The relatively thick black arrows for phosphagen kinase mediated diffusion indicates that the vast majority of net high-energy phosphate diffusion occurs in the form of phosphagen (AP or PCr), rather than directly as ATP (thin black arrows).

diffusion is faster in one direction than another (Fig. 3). Mathematical analyses of the time dependence of D in muscle indicate that it arises from intracellular structures that hinder molecular diffusion (Kinsey et al., 1999; de Graaf et al., 2000; Kinsey and Moerland, 2002). Therefore, since there is a greater likelihood of encountering a structure as the diffusing molecule travels over larger distances, D decreases as diffusion time increases until a lower, steady-state D is reached after about 100 ms (Fig. 3). This pattern is typical of diffusion through a porous medium, where the lower steady-state D is reached when the diffusion distance substantially exceeds the spacing between the structural barriers to diffusion in the medium [see Kinsey et al. (Kinsey et al., 1999) and references therein].

The time-dependent decrease in D is much more striking for radial diffusion across the fiber than for axial diffusion along the fiber length (anisotropy), indicating that intracellular barriers such as the myofibrillar array and the sarcoplasmic reticululum (SR) hinder radial diffusion in muscle to a greater extent than axial diffusion (Fig. 3). The anisotropic nature of diffusion has important implications, since it is radial diffusion (rather than axial diffusion) that is generally considered to be most relevant to energy metabolism in skeletal muscle. To evaluate the effect of the pattern of diffusion in muscle, it is instructive to evaluate the distance a diffusing molecule can traverse in a cell in a given amount of time. The average movement of a molecule (λ), termed the one-dimensional root-mean square displacement, can be calculated as $\lambda=\sqrt{2Dt}$, where t is the diffusion time. As D declines with increasing t, the distance covered by the molecule per unit time will decline. This can be seen in Fig. 3C,D for PCr diffusion in fish white muscle, where at short diffusion distances of a few μm, there is little effect of intracellular structures on net molecular transport. However, at diffusion distances above about 8 μm, there is a 140% increase in the time required to traverse a given distance. Thus, as diffusion distances between mitochondria increase, not only do molecules have a greater distance to travel, but they are moving at about half the rate as over short distances. This may contribute to diffusion limitation of metabolism even if diffusion distances are relatively short.

Muscle fiber size diversity

The prevalence of studies on mammalian systems has led to the commonly held notion that muscle fibers are small, usually ranging from 10 μm to much less than 100 μm in diameter. Presumably, cells are limited in size to promote short maximal intracellular diffusion distances. Short diffusion distances are necessary to facilitate both rapid O_2 flux to mitochondria and ATP flux from mitochondria to sites of ATP demand. Thus, a likely functional consequence of excessive cell size is a reduced capacity for critical oxidative metabolic processes (Egginton and Sidell, 1989; Egginton et al., 2000; Boyle et al., 2003; Johnston et al., 2003a; Johnston et al., 2004; Kinsey et al., 2005;

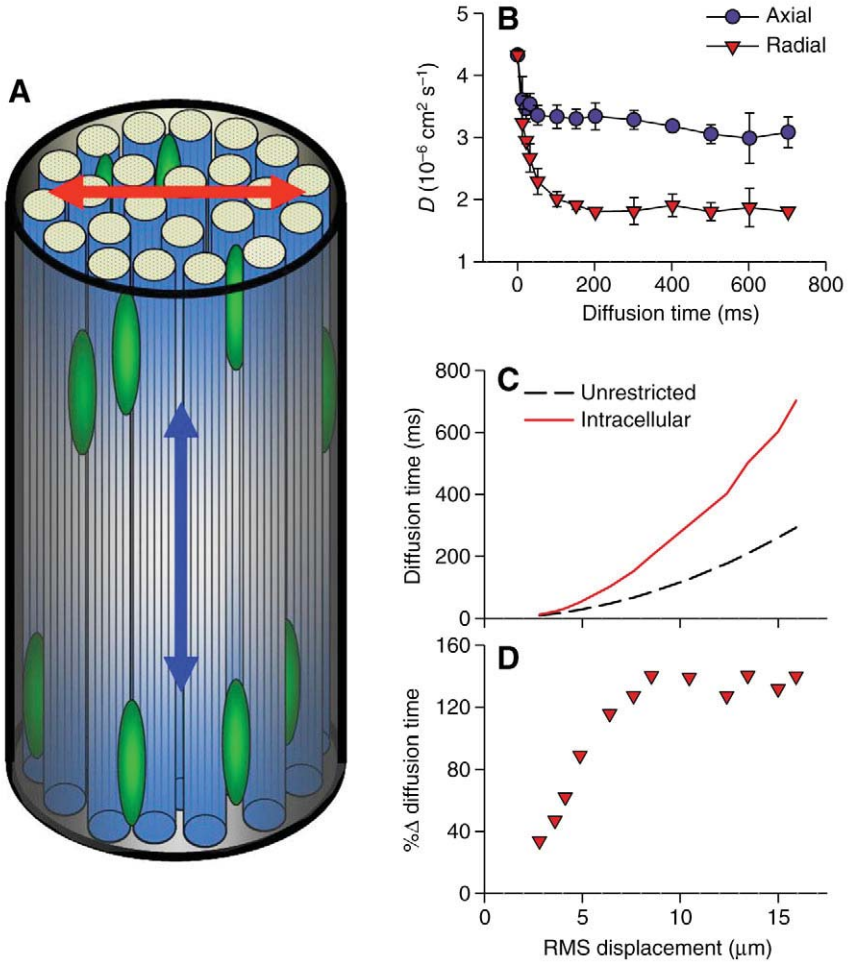

Fig. 3. The diffusion coefficient (D) is time- and orientation-dependent in muscle. (A) A 3-dimensional schematic of a muscle fiber shows the major barriers to diffusion, where radial diffusion (red arrow) is hindered both by the sarcoplasmic reticulum (blue shading), which is a partial membrane that surrounds myofibrils (small cylinders), and by the thick and thin filament lattice (thin black lines). In contrast, there are comparatively few barriers to axial diffusion (blue arrow), and elements that potentially limit axial diffusion, such as Z-disks (not shown), have little effect on D. Green spheroids represent mitochondria. (B) D of PCr in white muscle of goldfish is reduced from the value in solution (D of PCr in solution is shown at a diffusion time of zero) (Ellington and Kinsey, 1998), and declines to a steady state value that is lower for radial diffusion, due to the presence of intracellular barriers such as the sarcoplasmic reticulum [data from Kinsey et al. (Kinsey et al., 1999) ©1999, John Wiley and Sons, Ltd, reproduced with permission]. (C) Hindered radial diffusion in muscle (red line) increases the time required for PCr to diffuse a given distance compared to diffusion in water (broken black line). RMS is root mean square displacement and reflects the average movement of a molecule. (D) There is a large percent change in the radial diffusion time over the short intracellular diffusion distances that characterize most cells. Therefore, below a threshold diffusion distance of about 8 μm (time-dependent range), shorter diffusion distances can support higher metabolic rates without diffusion limitation.

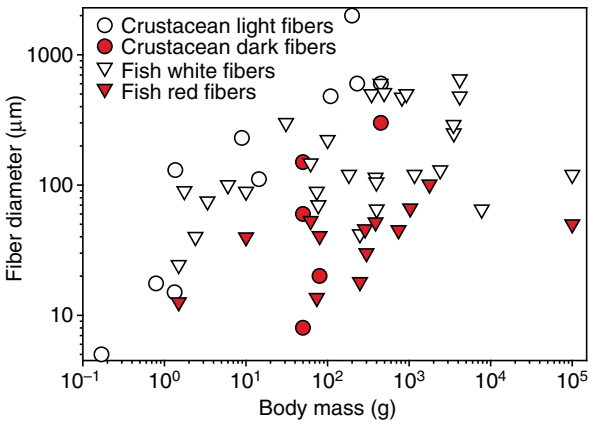

Fig. 4. Examples of fiber size diversity in crustaceans and fishes in anaerobic (white symbols) and aerobic muscle (red symbols). In some cases, body mass was estimated from length measurements using species-specific regression analysis. Data are from the published literature (Jahromi and Atwood, 1971; Johnston and Lucking, 1978; Weatherley et al., 1979; Silverman and Charlton, 1980; Weatherley and Gill, 1987; Johnston, 1982; Jones and Sidell, 1982; Tse et al., 1983; Hoyle et al., 1986; Hoyle, 1987; Egginton and Sidell, 1989; Londraville and Sidell, 1990; Archer and Johnston, 1991; Stokes and Josephson, 1992; Egginton et al., 2000; Boyle et al., 2003; Johnston et al., 2003a; Johnston et al., 2003b; Johnston et al., 2004; Nyack et al., 2007) and personal observations (S.T.K., unpublished results).

Hardy et al., 2006; Nyack et al., 2007). In addition, the pattern of diffusion in muscle described above indicates that even small fibers are likely to encompass diffusion distances that are long enough to lead to a dramatic reduction in D (Fig. 3).

Across the animal kingdom muscle fibers may greatly exceed the typical mammalian size range, and in crustaceans and fishes alone, fiber size spans >3 orders of magnitude (Fig. 4). Moreover, since the increase in skeletal muscle mass during development often occurs through an increase in fiber diameter and length (hypertrophy), rather than an increase in fiber number (hyperplasia), maximal intracellular diffusion distances often increase during development, and in some cases the increase may be dramatic. For instance, white muscle fibers from crustaceans and fishes are generally <30 μm in diameter in juvenile animals, while in some species fiber sizes in adult animals may be hundreds of μm (e.g. Jahromi and Atwood, 1971; Hoyle, 1987; Weatherley and Gill, 1987; Boyle et al., 2003; Johnston et al., 2003a; Johnston et al., 2003b; Johnston et al., 2004; Nyack et al., 2007). This is particularly prevalent in species that undergo a many-fold increase in body mass during development, such as the blue crab *Callinectes sapidus* (Boyle et al., 2003), or the black sea bass *Centropristis striata* (Nyack et al., 2007). Thus, aerobic metabolism in muscle may become increasingly constrained as the animals grow.

Solutions to 'big' problems

How then is function preserved during growth in organisms with muscle fibers that undergo a large increase in size? One strategy, which has been observed both in burst swimming muscle of blue crab (Boyle et al., 2003) and black sea bass (Nyack et al., 2007), entails the redistribution of intracellular metabolic machinery over the course of development. In the small fibers from juvenile animals of both species, mitochondria are predominantly distributed evenly throughout the fiber (intermyofibrillar mitochondria). However, as the animals grow and fiber diameter increases to several hundred μm, the mitochondria become increasingly clustered at the periphery of the cell (subsarcolemmal mitochondria) and are much less dense in the fiber interior. This ontogenetic redistribution of mitochondria reduces diffusion distances between blood and mitochondria, presumably to enhance O_2 flux into the cell. However, it comes at the expense of increased intracellular diffusive distances between mitochondrial clusters, which may greatly slow ATP diffusive flux (Boyle et al., 2003; Nyack et al., 2007). Kayar et al. (Kayar et al., 1986) argued that the predominance of mitochondria near the periphery of mammalian skeletal muscle fibers reflects the importance of short path lengths for O_2 and other blood-borne substrates from capillaries to mitochondria, relative to the path lengths for intracellular diffusion of high energy phosphate molecules between mitochondria and myofibrils. The large fibers of some crustaceans and fishes appear to be a more extreme example of this trade-off.

The impact of the shift in mitochondrial distribution during fiber growth can be demonstrated by rearranging the equation described above for displacement, λ, to solve for diffusion time, t, using time-dependent diffusion coefficients and mitochondrial spacing data from a crustacean muscle example (Kinsey and Moerland, 2002; Kinsey et al., 2005). In blue crab anaerobic muscle fibers, the combined effect of the changes in mitochondrial distribution and the time dependence of diffusion (Fig. 3) is that metabolites can traverse half the distance between mitochondria in the small fibers of juveniles in <20 ms (λ=2.7 μm and D=2×10^{-6} cm^2 s^{-1} for short distance diffusion), whereas in the large fibers of adults diffusion between mitochondrial clusters at the periphery of the fiber takes 7.5 min (λ=300 μm and D=1×10^{-6} cm^2 s^{-1} for long distance diffusion), or 22 500 times longer!

The burst fibers described above rely on endogenous fuels that are present throughout the cell, such as AP in crustaceans and PCr in fishes and other vertebrates, as well as glycogen, to power a series of rapid contractions. The anaerobic contractile process is therefore not dependent on transport of either O_2 to the mitochondria or ATP/phosphagen from mitochondria to cellular ATPases. Thus, it would not be expected that contractile function in these fibers is influenced by an increase in fiber size or changing mitochondrial distribution. However, large fiber size might be expected to impact the rate of metabolic recovery after a series of burst contractions. This may have serious implications for the animal's survival if multiple bouts of high-force contractions are needed, such as during repeated predator–prey interactions. Crustaceans appear to compensate for large fiber size by relying on anaerobic metabolism to accelerate key phases of post-contractile recovery, such as phosphagen resynthesis (e.g. Henry et al., 1994; Johnson et al., 2004). We have observed that post-contractile AP recovery in large burst fibers that power swimming in blue crabs is twice as fast as would be expected from indices of aerobic capacity alone (Kinsey et al., 2005). Further, this recovery is associated with both significant lactate accumulation (Johnson et al., 2004)

and glycogen depletion (Boyle et al., 2003) in large fibers of adults, but not the small fibers of juveniles. While this metabolic strategy will ultimately put the animal further in O_2 debt, it serves the more immediate need of facilitating a faster recovery between burst contractions.

While the compensations for large fiber size described above refer to muscles that contract anaerobically, there are also large fibers that are used for aerobic, steady-state locomotion (Fig. 4). An unusual example is again found in the muscles that power the paddle-like swimming legs of the blue crab, which is both an excellent burst and endurance swimmer. Endurance swimming recruits dark muscle fibers (so-called because of the brown color that results from a high density of mitochondria; they are not red in color like some other aerobic fibers because they lack myoglobin). These dark fibers also grow hypertrophically and reach the same large size in adult animals as the anaerobic fibers. However, the secondarily evolved aerobic contractile function of these muscles should favor a small fiber size throughout development. To accommodate the conflicting demands for hypertrophic growth and aerobic function, the dark fibers have small, mitochondria-rich subdivisions (Tse et al., 1983). These fibers form new subdivisions continuously during fiber growth, so subdivision size is independent of animal body mass, and they also have evolved intra-fiber perfusion pathways to facilitate O_2 delivery to the subdivisions (Johnson et al., 2004). Thus, blue crab dark fibers are unusual in having a metabolic functional unit (subdivision) that retains small dimensions (~35 μm) throughout post-metamorphic development, and an apparent contractile functional unit (fiber) that grows hypertrophically to extreme proportions (>600 μm) (Hardy et al., 2006).

Not so fast!

The results described above led us to the hypothesis that aerobic metabolic processes would be limited by intracellular metabolite diffusion in large burst contraction fibers, which have large distances between mitochondrial clusters, but not in aerobic fibers, which do not. This argument was based on the seemingly reasonable, but in hindsight naïve, notion that extreme diffusion distances alone present a formidable obstacle to muscle metabolic function. Coupling experimental data with a reaction–diffusion mathematical model, however, we found that the observed rate of AP resynthesis following burst contraction in the anaerobic locomotor fibers of small and large blue crabs was not dramatically limited by intracellular metabolite diffusion (Kinsey et al., 2005). That is, the rate of post-contractile AP resynthesis was too slow to be substantially limited by intracellular metabolite diffusion. This finding held even in the most extreme case, when we assumed that the large fibers, which had a mean diameter of nearly 600 μm, had exclusively subsarcolemmal mitochondria (and therefore a diffusion distance of 300 μm). Since these experiments were conducted *in vivo*, the effects of transport of both O_2 (which was not modeled) and high-energy phosphate molecules would be manifested. The fact that metabolite diffusion did not impact AP recovery suggested that the responses to developmental increases in fiber size, including the shift in mitochondrial distribution and the reliance on anaerobic metabolism to accelerate recovery, may compensate more for O_2 flux limitations.

To further explore this process, we took advantage of certain properties of the white muscle fibers from the black sea bass, which grow to similar dimensions as the blue crab fibers. Unlike blue crab fibers, however, the white muscle fibers from fish can be activated electrically by field stimulation, and fish muscle, like other vertebrates, is not known to produce lactate after contraction (Curtin et al., 1997). We therefore used small *ex vivo* preparations of white muscle fiber bundles (1 mm diameter) in a superfusion medium of high oxygen partial pressure (P_{O_2}), and performed burst contraction–recovery experiments similar to those described above. The high P_{O_2} ensured adequate O_2 flux to the core of the fiber bundle, which effectively removed the influence of O_2 diffusion and allowed us to focus exclusively on the extent to which intracellular metabolite diffusion limited post-contractile PCr recovery (Nyack et al., 2007). In addition, the absence of anaerobic metabolism following burst contraction in the fish muscle eliminated a potentially confounding contributor to the recovery rate. Consistent with the earlier study, reaction–diffusion modeling showed that the observed PCr recovery rate in these burst fibers was not fast enough to be dramatically limited by intracellular metabolite diffusion (Nyack et al., 2007). Further, the PCr recovery rate in muscle from fish that ranged in body mass over 3000-fold was nearly proportional to the mitochondrial density. Thus, the low mitochondrial density in these white fibers was primarily responsible for the low recovery rate, and intracellular diffusion played only a modest role in modifying the rate.

The experiments described above indicate that very large diffusion distances do not necessarily imply diffusion limitation; the rate of ATP turnover interacts with diffusion in a complex manner. However, further simulations showed that enhancing the rate of ATP production does lead to increased control of aerobic flux by diffusion (Kinsey et al., 2005). In this sense, diffusion places constraints on the evolution of muscle design. In an effort to better understand the effect of ATP turnover rate, we examined contraction–recovery in the aerobic locomotor muscles of blue crabs, which have a high capacity for ATP turnover but short diffusion distances (due to the intracellular subdivisions described above). Although post-contractile AP recovery was faster in these highly aerobic fibers than observed in the prior studies, mathematical modeling again indicated that metabolite diffusion only slightly altered the observed recovery rate (Hardy et al., 2006). In this study, a burst contraction–recovery procedure was employed as before because it is a tractable experimental model. However, during the aerobic, steady-state contractions for which these muscles are designed, ATP turnover rates may be considerably higher. When we mathematically simulated reasonable rates of ATP turnover during steady-state contraction in these fibers, we found sizable concentration gradients within the fiber and evidence for substantial limitation by intracellular metabolite diffusion (Hardy et al., 2006).

On the brink: diffusion limits on aerobic design

The experiments described above, combined with mathematical analyses of the observed process rates, reinforce the notion that the extent of diffusion limitation depends on a non-linear interaction of diffusion distance and metabolic rate. This is consistent with the pattern, long recognized in muscle,

that aerobic fibers are generally smaller than anaerobic fibers. But how close are fibers to being substantially limited by diffusion, and how might diffusion influence muscle design? To address this question we developed a reaction–diffusion model that was intentionally simpler than our prior efforts so that it could be broadly applied to a diversity of muscle types. The model includes linear expressions for mitochondrial ATP production, with a rate dependent on [ADP], and cellular ATP consumption, with a rate dependent on [ATP]. ATP production and consumption are linked by steady-state species conservation equations. These equations ensure mass balance throughout the modeled domain and define diffusion and reaction for each metabolite over a range of ATP turnover rates and spatial scales. Metabolite concentration profiles were examined for each simulation condition to ensure that steady state was achieved without fully depleting ATP. One key aspect of the model is that the mitochondrial production of ATP is dependent on the delivery by diffusion of ADP back to the mitochondria in the reaction–diffusion cycle.

Fig. 5 demonstrates the interaction between ATP turnover rate, diffusion distance, and the effectiveness factor (η). The effectiveness factor is an index of diffusion limitation, where η is the ratio of the observed reaction rate (total integrated rate over the length scale) in the presence of diffusion to the reaction rate if diffusion were not limiting (as if the diffusion distance were zero) (Weisz, 1973). The denominator in this ratio can be thought of as the intrinsic reactivity of the system. Thus, when η=1 there is no diffusion limitation, whereas when η=0.5 the observed rate is half what it would be if diffusion were not limiting. Overlaid on the model surface are examples of muscles that range in design from very high aerobic ATP turnover rates and short diffusion distances (insect flight muscle) to very low aerobic ATP turnover rates and long diffusion distances (blue crab light levator muscle). In addition, developmental trajectories of some of the fibers that we have examined in our lab are indicated. It is interesting that many fibers appear to be only modestly limited by diffusion (η>0.75), but as noted for other biological systems (Weisz, 1973), they are at the brink of extreme diffusion limitation, meaning that further increases in ATP turnover rate or diffusion distance may lead to a large decrease in η.

If fibers often have ATP turnover rates and diffusion distances that approach substantial diffusion limitation, this means that there is a large 'safe' region of the energetic surface in Fig. 5, where $\eta \cong 1$, that is not exploited in many adult animals. This may be partly explained by the mutually exclusive nature of muscle design. The proportion of total muscle fiber volume allocated to myofibrils, SR and mitochondria is governed by functional demands (e.g. Rome and Lindstedt, 1998). Thus, an anaerobic burst contractile muscle will devote most of its volume to myofibrils and SR, and very little to mitochondria. This will necessarily lead to longer diffusion distances between mitochondria and a low rate of aerobic ATP turnover, placing the fiber on the left side of the surface in Fig. 5. In highly aerobic

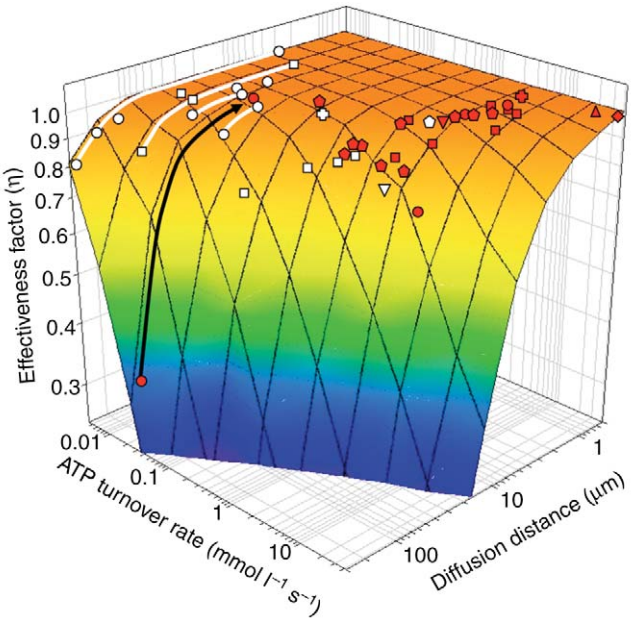

Fig. 5. The interaction of ATP turnover rate, diffusion distance and the effectiveness factor (η), which is the ratio of the reaction rate in the presence of diffusion to the rate if diffusion were not limiting. The surface was generated from a Loess fit to output from a simplified reaction–diffusion model with linear expressions for ATP production (mitochondria), ATP consumption (ATPases) and diffusion (see text for additional details). The symbols represent the positions on the surface of a variety of anaerobic (white) and aerobic (red) muscle fibers from insects (bee flight muscle) (diamonds), crustaceans (circles), fishes (squares), amphibians (frog lumbrical muscle) (inverted triangle), reptiles (including rattlesnake tailshaker muscle) (crosses), bird (hummingbird flight muscle) (triangle), and mammals (pentagons). The white lines are developmental trajectories from individual species for white muscle fibers that grow hypertrophically (muscles are, in order from low to high ATP turnover rate: blue crab light levator, black sea bass white epaxial muscle, pink shrimp abdominal flexor and grass shrimp abdominal flexor). The black arrow indicates the impact of subdividing the large aerobic fibers of blue crabs, which greatly reduces the intracellular diffusion distances and alleviates diffusion limitation associated with the hypothetical, non-subdivided case (value with a low η) (see text for additional details). Diffusion distances were taken from direct measurements or calculated as in Tyler and Sidell (Tyler and Sidell, 1984) from the mitochondrial volume density and mitochondrial surface density [the latter was calculated assuming a mitochondrial surface area to volume ratio of 6 if not directly measured (Tyler and Sidell, 1984)]. ATP turnover rates per volume of muscle fiber were determined from direct measurements of O_2 consumption in tissue, isolated fibers, or isolated mitochondria assuming 22.4 l O_2 mol^{-1} O_2, an ATP/O_2 ratio of 6, and an intracellular water content that was 70% of wet mass. For cases where measurements of O_2 consumption were unavailable, ATP turnover rate was estimated from mitochondrial volume density assuming a sustainable rate of O_2 consumption of 3 ml min^{-1} cm^{-3} of mitochondrial volume [(Schwerzmann et al., 1989); this value was not applied to mitochondria with known high cristae surface densities]. Data are from the published literature (Tyler and Sidell, 1984; Andersen and Saltin, 1985; Egginton and Sidell, 1989; Schwerzmann et al., 1989; Stokes and Josephson, 1992; Curtin et al., 1997; Conley and Lindstedt, 1998; Johnston et al., 1998; Suarez, 1998; Egginton et al., 2000; Vicini and Kushmerick, 2000; Kanatous et al., 2002; Boyle et al., 2003; Kindig et al., 2003; Johnson et al., 2004; Stary et al., 2004; Kinsey et al., 2005; Hardy et al., 2006; Nyack et al., 2007) and personal observations (S.T.K., unpublished results). In studies of ectotherms from different temperature regimes, only data from the warm acclimated or adapted groups were included.

fibers, a much greater percentage of the fiber volume will be devoted to mitochondria, leading to relatively short diffusion distances and a high rate of aerobic ATP turnover, placing the fiber on the far right of Fig. 5. In this sense, diffusion constraints contribute to the mutually exclusive design of muscle described elsewhere (Rome and Lindstedt, 1998).

In many cases, however, space allocation constraints may not fully explain the observed pattern of diffusion limitation in fibers. For instance, the positions of many of the fibers in Fig. 5 are based on conservative estimates of diffusion distance, derived from total mitochondrial fractional volume. These estimates therefore assume a uniform distribution of mitochondria across the fiber and no impact of fiber size. However, mitochondria are typically not homogeneously distributed, and in fibers with predominantly subsarcolemmal mitochondria, intracellular diffusion distances may approximate the fiber radius. This is the case for blue crab light levator muscle and black sea bass white epaxial muscle, where metabolite diffusion only becomes somewhat limiting in the large fibers of adult animals, which are at the precipice of a dramatic reduction in η. Further, increased fiber size will lead to longer pathways for O_2 diffusion, which may reduce O_2 flux to the core of the cell. If so, this could result in less active mitochondria in the core of the fiber, leading to an increase in the effective intracellular distance between the more active mitochondria at the fiber periphery. This effect was noted by Mainwood and Rakusan, who mathematically examined the interaction of fiber size, O_2 flux and metabolite flux (Mainwood and Rakusan, 1982). These authors suggested that maximal aerobic flux could be attained by minimizing diffusion distances for O_2 by clustering mitochondria at the periphery of the fiber, at the expense of longer diffusion distances between mitochondria for ATP-equivalent diffusion. Hogan et al. recently demonstrated that rates of mitochondrial NAD(P)H oxidation were reduced in the core of isolated *Xenopus* fibers under physiological realistic extracellular P_{O_2} conditions (Hogan et al., 2005). This lends experimental evidence to the notion that in working muscle, interior mitochondria may be less active than more peripheral mitochondria, meaning that the effective diffusion distance for ATP-equivalents may be greater than would be expected based on mitochondrial volume density alone. Thus, it is likely that inhomogeneous mitochondrial distribution and fiber size strongly influences aerobic metabolic design. It is also possible that many fibers are closer to substantial diffusion limitation than indicated by the analysis in Fig. 5.

One implication of the above argument is that fibers are often larger than they need to be. Why might fibers be as large as possible? One plausible explanation is that relatively large fibers arise simply because hypertrophy often accounts for much of the increase in muscle mass during animal growth, leading to very large fiber size in species that undergo a dramatic increase in body mass. In this case, fiber growth would have to be moderated if there were selective pressure for relatively high rates of ATP turnover, as would be expected in species that attain large adult body masses and have high activity levels. Thus, in some species the contributions of hypertrophy and hyperplasia to muscle growth may depend on the extent of diffusion limitation in the adult animal (Fig. 5) (Johnston et al., 2004). This may explain why there is not always a clear relationship between fiber size and body mass (Fig. 4).

It is also possible that there is positive selective pressure that favors relatively large fibers. Johnston et al. (Johnston et al., 2003a; Johnston et al., 2004) proposed the 'optimum fiber size' hypothesis for cold-water fishes, which often have large muscle fibers. These authors postulated that fibers will be as large as possible without incurring diffusion limitation in order to minimize the sarcolemmal membrane area over which membrane potential must be maintained. Thus, the costs of ionic homeostasis, which can be a considerable fraction of basal metabolic rate, would be reduced. A low sarcolemmal surface density may also promote fatigue resistance, since fatigue is often associated with ionic imbalances such as an accumulation of extracellular potassium ions (Fitts, 1994), and ionic exchange per volume of muscle may be reduced in muscles composed of relatively large fibers. Presently, however, it is unclear whether selection is responsible for pushing many fibers to be near diffusion limitation. It is also likely that any influence that fiber size exerts on aerobic design can be altered by other factors, such as body temperature, metabolic compensations, perfusion rates, blood P_{O_2}, cellular lipid content, and the presence/absence of myoglobin.

Conclusions

Intracellular diffusion of ATP-equivalents is a fundamental component of aerobic metabolism, but it is often neglected because diffusion distances are usually assumed to be short. However, the full spectrum of skeletal muscle design found in animals encompasses diffusion distances and ATP turnover rates that vary over several orders of magnitude. Analysis of the non-linear interaction of ATP turnover rate with diffusion distance indicates that what may appear to be excessively long diffusion distances in certain fiber types, in fact do not greatly compromise aerobic metabolic rate. Similarly, the presence of relatively short diffusion distances does not necessarily imply that diffusive flux has no influence on metabolic rate. While diffusion limitation *per se* appears to be relatively minor, metabolite diffusion may play an important role in shaping the evolution of fiber design. Many species have fibers that appear to be at the brink of substantial diffusion limitation at some stage of development. Since intracellular diffusion distance is often related to fiber size, this implies that many fibers may have evolved to be as large as possible without incurring diffusion limitation, as proposed by Johnston et al. (Johnston et al., 2003a; Johnston et al., 2004).

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