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Review

Shaken and stirred: muscle structure and metabolism

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Summary

Muscles are ideal models with which to examine the relationship between structure and metabolism because they are some of the most highly structured cells, and are capable of the largest and most rapid metabolic transitions as well as the highest metabolic rates known. Studies of metabolism have traditionally been conducted within what can considered as the kinetic paradigm provided by 'solution biochemistry'; i.e. the rates of enzymatic reactions are studied in terms of their regulation by mass-action and allosteric effectors and, most recently, metabolic control analysis of pathways. This approach has served biology well and continues to be useful. Here, we consider the diffusion of small and large molecules in muscles and energy metabolism in the context of intracellular space. We find that in attempting to explain certain phenomena, a purely kinetic paradigm appears insufficient. Instead, phenomena such as the 'shuttling' of high-energy phosphate donors and acceptors and the binding of metabolic enzymes to intracellular structures or to each other are better understood when metabolic rates and their regulation are considered in the context of intracellular compartments, distances, gradients and diffusion. As in all of biology, however, complexity dominates, and to such a degree that one pathway may consist of several reactions that each behave according to different rules. 'Soluble' creatine kinase operates at or near equilibrium, while mitochondrial and myofibrillar creatine kinases directly channel substrate to (or from) the adenine nucleotide translocase and actomyosin-ATPase, their operation being thus displaced from equilibrium. Hexose 6-phosphate metabolism appears to obey the rules of solution biochemistry, e.g. phosphoglucoisomerase behaves as Haldane would have predicted in 1930. In contrast, given low steady-state substrate and product concentrations and high flux rates, a number of glycolytic reactions further downstream must be catalyzed by enzymes localized in close proximity to each other. Metabolites may be channeled within these complexes. When observed, mechanistic differences between species in the same steps or processes should not be surprising, considering how animals vary so much in structures, mechanical properties, mitochondrial contents and metabolic rates. This analysis suggests that declarations of the triumph of one mechanism or paradigm over all others, as well as calls for the abandonment of solution biochemistry, are unwarranted. Rather, metabolic biochemistry would seem better served by reconciling the old and the new.

Key words: muscle structure, metabolism, intracellular, myofibril, diffusion, glycolysis, channelling, kinetics, regulation, creatine kinase.

Introduction

Muscle fibers, specialized for the conversion of chemical energy into mechanical work, are some of the most highly structured cells known. Muscles perform diverse mechanical functions, ranging from the maintenance of posture and the propulsion of blood, to the movement of limbs for locomotion. They display the largest changes in metabolic rate when undergoing transitions between rest and exercise, as well as the highest metabolic flux rates known in the animal kingdom. From these perspectives, muscles are ideal models for probing the relationships between cell structure and metabolism.

In cardiac and locomotory muscles, the transition from rest to exercise is accompanied by an increase in the rate of cellular ATP hydrolysis, brought about by the activation of actomyosin ATPase and, to a variable extent, Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase. Bioenergetic pathways are regulated such that ATP is synthesized at rates that match hydrolysis rates. The stoichiometric matching of rates of synthesis and hydrolysis allows the maintenance of contractile function. Bouts of exercise may vary in both duration and intensity, and many species of animals possess fiber types specialized in structure and biochemical properties to serve their particular needs. Brief (e.g. 1–2 s) bouts of exercise may be accompanied by the

hydrolysis of relatively small amounts of ATP. Under these circumstances, ATP concentration is maintained (over time, thus, the term 'temporal buffering') at the expense of creatine phosphate (CrP) in vertebrates or arginine phosphate (or other 'phosphagens') in the invertebrates, *via* reactions catalyzed by creatine kinase (CK) or other phosphagen kinases. High-intensity, 'burst' exercise, sustainable for up to several minutes in some species, involves the recruitment of muscles with low oxidative but high glycolytic capacities. Under these conditions, ATP is derived primarily from glycolysis, and the flux rate from glycogen to lactate may increase up to several hundredfold higher than at rest. During prolonged, steady-state exercise performed by hearts and aerobic locomotory muscles, the energy requirements are met mainly by mitochondrial oxidative phosphorylation (Suarez, 1996).

Muscle fibers appear to be crammed full of myofibrils, enzymes, mitochondria, nuclei and intracellular membranes. It is appropriate to begin this analysis by considering whether there are functional consequences to accommodating these components in various proportions.

Intracellular space and muscle design

An organelle's perspective

We consider here muscles designed for aerobic, steady-state work involving repetitive cycles of contraction and relaxation as hearts or mantle muscles pump blood or water, and as legs, fins or wings are used in long-distance running, swimming or flying. Muscles performing steady-state work tend to operate at characteristic optimal frequencies. Small hearts beat faster than larger ones, and stride, wingbeat and tailbeat frequencies generally increase with declining body size. Pennycuick and Rezende (1984) present a simple (but for our purposes, sufficient) way to consider how the mechanical performance of muscle relates to the metabolic, energy-supplying machinery. The average volume-specific power output, P_v, of muscles doing steady-state work is:

$$P_v = srf\,, \tag{1}$$

where stress s is the force/cross sectional area, strain r is the fractional change in length per contraction, and f is the operating frequency. If the simplifying assumption is made that, across species, s and r do not vary greatly among muscles of a given type, then P_v is directly proportional to, and largely determined by f, the operating frequency. Therefore, rates of ATP hydrolysis are determined by f. Muscles that have evolved to operate at high frequencies for extended periods require high capacities for aerobic ATP synthesis and, therefore, high volume densities (i.e. fraction of cell volume) of mitochondria. In the flight muscles of hummingbirds and insects, mitochondrial volume densities are as high as 35% and 45%, respectively (Suarez, 1996). Among synchronous muscles, high operating frequencies require high rates of release and reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR). Thus, Ca^{2+}-ATPase activities (Blank et al., 1989) and SR volume densities (fraction of cell volume occupied by SR) increase with f (Appelt et al., 1991; Josephson and Young, 1987; Rome and Lindstedt, 1998). However, many species of flying insects possess asynchronous muscles that are characterized by a poorly developed SR, so that higher frequencies are associated with higher mitochondrial, but not SR, volume densities (Josephson et al., 2000).

At a given cell volume, fractional volumes of mitochondria and SR can only increase at the expense of sarcomere volume. Thus, with increasing operating frequency and the accompanying increases in capacities for Ca^{2+} cycling and oxidative phosphorylation, P_v would be expected not to increase linearly with f, but instead to reach an asymptote and to decline (Pennycuick and Rezende, 1984). Theory suggests, therefore, that availability of intracellular space should impose limits upon the enhancement of capacities for Ca^{2+}cycling and aerobic ATP synthesis. This is, in fact, observed in cardiac and locomotory muscles, where mitochondrial volume densities generally do not exceed about 45% (Suarez, 1996); higher values are found in highly modified muscles that perform no mechanical work (e.g. billfish heater organs; Block, 1991).

Molecules' eye views of sarcoplasm

The main structural features of striated muscle cells are well known to most biologists. Besides the well-known actin and myosin filaments and their associated proteins, muscles contain a membrane cytoskeleton as well as a longitudinal cytoskeletal lattice. The proteins of the *M* and *Z* lines act as structural integrators that hold other proteins in place and specify sarcomere length. Thus, the sarcoplasm contains structural proteins of various thicknesses and lengths, arranged in patterns ranging from highly ordered and regular to irregular and mesh-like. In the midst of these fibrous proteins, SR and mitochondria, are low molecular mass metabolites, globular proteins and enzymes of various shapes and sizes, diffusing and performing their functions (Fig. 1). How do the structural features of the sarcoplasm influence diffusion rates and how do diffusion rates influence metabolism?

A small molecule's perspective

In general, intracellular diffusion can be retarded by the (1) viscosity of the fluid medium, (2) crowding and (3) binding of molecules to intracellular structures (Verkman, 2002). The viscosity of the sarcoplasm of skeletal muscle fibers is not very high, and the resulting effect on the diffusion of high-energy phosphate compounds is relatively modest. Using pulsed gradient ^{31}P-NMR spectroscopy and restricting measurements to short diffusion times, Hubley et al. (1995) determined that the radial diffusion coefficients of ATP and CrP (D_{ATP} and D_{CrP}, respectively) are 30% and 34% less than in aqueous solutions of similar ionic composition. D_{ATP} and D_{CrP} values approximately 50% lower than in water were obtained by Yoshizaki et al. (1990), values consistent with slightly higher estimates of sarcoplasmic viscosity (approximately 2.3 times that of water; Arrio-Dupont et al., 1996). The intracellular diffusion of low-molecular mass metabolites in skeletal

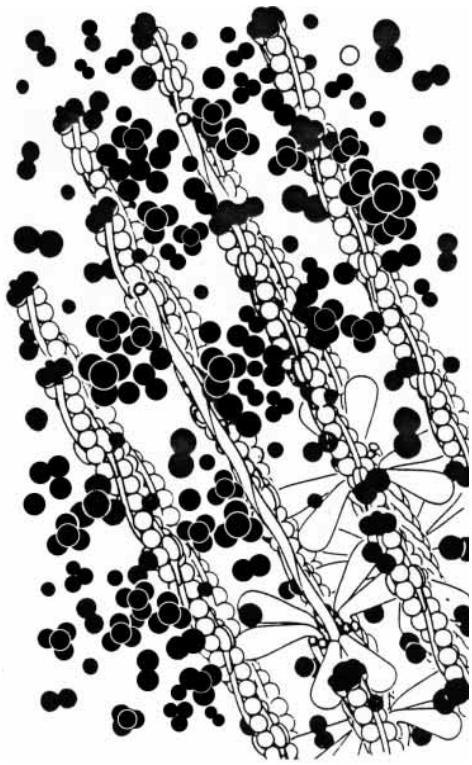

Fig. 1. Cartoon of sarcoplasmic space showing actin and myosin filaments (white), and glycolytic enzymes (black) drawn to scale and at their approximate intracellular concentrations (redrawn and modified from Maughan and Wegner, 1989).

muscles, studied using pulsed gradient ^{31}P-NMR spectroscopy, is both time- and orientation-dependent. Kinsey et al. (1999) found that in fast-twitch glycolytic (white) fibers, D_{CrP} values for radial diffusion declined over time, whereas values for axial diffusion were only slightly affected. In slow-twitch oxidative (red) fibers, D_{CrP} values for both radial and, to a lesser degree, axial diffusion declined with time. It was inferred from these results that, although CrP diffusion is not impeded by myofilaments, the SR and mitochondria act as physical barriers to its diffusion. The low mitochondrial volume density of white fibers result in the SR being the only significant barrier (to radial diffusion), while the presence of significant volume densities of both SR and mitochondria in red fibers result in the time-dependent decline of both radial and axial D_{CrP} values. Nevertheless, these D_{CrP} values declined by only 50% and are still 2–3 orders of magnitude greater than cytoplasmic diffusion coefficients (D_{cyt}) of macromolecules.

A large molecule's perspective

The diffusion of macromolecules in muscles has been studied using a variety of techniques, including measurement of the rate of glycolytic enzyme leakage from mechanically skinned fibers (Maughan and Lord, 1988; Maughan and Wegner, 1989), as well as microinjection of myoglobin (Mb) into muscle fibers and micro-spectrophotometry to measure time-dependent changes in Mb absorbance (Papadopoulos et al., 1995). Alternatively, cytoplasmic diffusion coefficients (D_{cyt}) of inert, fluorescently labeled macromolecules or proteins of various molecular masses have been estimated. In one such study (Arrio-Dupont et al., 1996), fluorescein isothiocyanate-labeled dextrans of various molecular masses were microinjected into cultured myotubes, and D_{cyt} values estimated by a photobleaching technique. Diffusion coefficients in aqueous medium (D_w) were estimated in parallel experiments. As in the case of ATP and CrP, D_{cyt} values for dextrans are consistently lower than D_w. Dextrans in aqueous solution, however, assume random-coil conformations and do not behave as compact spheres, and their hydrodynamic radii are higher than those of globular proteins of equal molecular mass. Taking this into account, relative diffusion coefficients (D_{cyt}/D_w), plotted against hydrodynamic radii R_h, decline with increasing R_h. These results compare favourably with predictions of model calculations that take into account both the crowding effect of soluble proteins in the fluid medium and screening effect of myofilaments (Arrio-Dupont et al., 1996).

In contrast with dextrans, globular proteins behave more like rigid spheres in solution. Using the same model system and experimental approach, Arrio-Dupont et al. (2000) estimated D_{cyt} values of proteins of a wide range of molecular mass. As with the dextrans, D_{cyt}/D_w values decline with increasing R_h. However, protein diffusivities decline more rapidly than those of dextrans. The proteins used were selected partly because they were assumed not to participate in specific binding interactions. However, if similar-sized proteins formed complexes with other globular proteins, their D_{cyt}/D_w values would be much lower. Protein complexes of molecular mass approximately 500 kDa, or higher, are virtually immobile in the sarcoplasm (Arrio-Dupont et al., 2000). Fig. 2 presents D_{cyt} values *versus* molecular masses of several globular proteins obtained using cultured myotubes (Arrio-Dupont et al., 2000) and from adult, skinned fibers (Maughan and Lord, 1988; Maughan and Wegner, 1989). The latter authors obtained their estimates by measuring the rates of leakage of glycolytic enzymes from skinned fibers. D_{cyt} values obtained by this approach are generally lower than those measured using fluorescently labeled proteins injected into cultured myotubes (Fig. 2). Although this might be due to structural differences between the cells, as suggested by Arrio-Dupont et al. (2000), it is difficult to exclude methodological differences (e.g. enzymes may somehow be more greatly inhibited from diffusing *out of*, rather than *within*, muscle fibers). Specific binding interactions (e.g. formation of complexes with other globular proteins, binding to fibrous sarcoplasmic proteins or proteins on the outer mitochondrial membrane) may have also occurred in the adult muscle fibers.

Enzymes and metabolism in sarcoplasmic space

A biochemist's perspective: disequilibrium in the field?

Myofibrils, mitochondria and SR in muscles appear so tightly packed that when the late Paul Srere examined electron micrographs of hummingbird flight muscle (seen in Suarez et

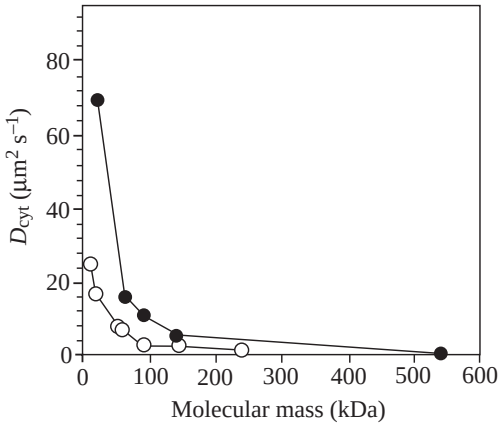

Fig. 2. Diffusion coefficients, D_{cyt}, of globular proteins spanning a range of molecular mass (redrawn and modified from Arrio-Dupont et al., 2000). Included are data obtained using cultured myotubes microinjected with fluorescently labeled proteins (Arrio-Dupont et al., 2000) (filled circles) and data obtained by measurement of the rates of diffusion of enzymes out of skinned, adult fibers (open circles) (Maughan and Lord, 1988).

al., 1991), he asked, rhetorically, 'where is the cytosol?' Biochemists have, over the course of a century of research, built up a mass of empirical data and mechanistic concepts largely based upon the assumption that most metabolic pathways consist of enzymes, substrates, products, mass-action and allosteric effectors that occur in solution. Life is studied, within this 'kinetic paradigm', in terms of the reactions involved in energy transformation, metabolic flux rates and their regulation. However, biochemists are now confronted with data that allow no escape from the fact that cells are highly structured and crowded, and that metabolism occurs, not just in time, but also in space. Textbook accounts of thermodynamics and enzyme kinetics provide what some now consider to be an insufficient paradigm for cellular metabolism. As a research discipline, metabolic biochemistry has not yet 'come to equilibrium' in attempting to reconcile reaction mechanism, kinetics and regulation with cell structure.

Consider a sequence of two enzyme-catalyzed reactions occurring by Michaelis–Menten kinetics, as:

$$E_1 + S \rightarrow E_1\text{–}S \rightarrow E_1\text{–}P \rightarrow E_1 + P$$

and

$$E_2 + P \rightarrow E_2\text{–}P \rightarrow E_2\text{–}Q \rightarrow E_2 + Q \ , \qquad (2)$$

where E_1 is enzyme 1, S and P are substrate and product, respectively, and freely diffusing P serves as the substrate for the next enzyme (E_2) in a pathway, leading to production of product (Q). It is thought that some reactions occur, instead, as

$$E_1 + S \rightarrow E_1\text{–}S \rightarrow E_1\text{–}P \ ,$$

$$E_1\text{–}P + E_2 \rightarrow E_1\text{–}P\text{–}E_2 \rightarrow E_1\text{–}Q\text{–}E_2$$

and

$$E_1\text{–}Q\text{–}E_2 \rightarrow E_1 + Q\text{–}E_2 \ , \qquad (3)$$

such that, after its formation, P does not dissociate from E_1, but is handed directly to E_2 within a complex consisting of E_1–P–E_2. Such a direct-transfer mechanism has been termed 'metabolic channeling', and the mechanism outlined above might be characterized as a 'perfect channel' if no P 'leaks' into bulk solution. An example of a 'leaky channel' is where E_1 and E_2 might be situated so close to each other that, although P diffuses (leaks) into the bulk medium, P from E_1 is used preferentially by E_2 over P in the bulk medium to produce Q. The significance of the selectivity of E_2 with respect to where P originates is apparent when one considers that many metabolites, e.g. ATP, are synthesized *via* reactions occurring at multiple sites in the cell. Although there is certainly evidence indicating channeling in some reaction sequences [e.g. channeling of oxaloacetate from malate dehydrogenase to citrate synthase in the Krebs cycle (Sumegi et al., 1990), ATP from the mitochondrial adenine nucleotide translocase to hexokinase (de Cerqueira Cesar and Wilson, 1998), ATP from myofibrillar CK to actomyosin-ATPase (Arrio-Dupont, 1988)], whether the phenomenon is indeed as ubiquitous as claimed by its proponents remains an open question. Much has been written concerning the functional significance of channeling, but some of these claims have been challenged. How much of the metabolic flux measured at any reaction actually occurs *via* channeling *in vivo*? What does the phenomenon of channeling imply concerning the applicability of enzyme kinetics and thermodynamics to the *in vivo* situation? Although now more than a decade old, articles in an entire issue of the *Journal of Theoretical Biology* (Cornish-Bowden, 1991) devoted to the channeling controversy still seem to accurately reflect the current state of disequilibrium of the field with respect to these issues.

A simple thought experiment

If metabolic enzymes were all 'soluble' but diffuse at rates 2–3 orders of magnitude more slowly than their substrates and products, they can be imagined, for the sake of argument, to be uniformly distributed in three-dimensional space and to move in slow motion relative to their more rapidly diffusing substrates and products. As enzyme E_1 converts S to P, let us suppose that a gradient is formed in which [P] is highest proximal to E_1 and its concentration declines with distance *d* (Fig. 3). E_2 is some distance from E_1 and catalyzes the formation of P to Q at a rate based on its kinetic properties and on the local [P]. This also leads to a gradient, with [Q] being highest in the vicinity of E_2. E_2 serves as a sink for P and, therefore, contributes to maintaining the gradient in [P]. If E_1 and E_2 were closer to each other, E_2 could operate at a higher fractional velocity. The extreme case of this would be seen if the two enzymes formed the complex E_1–E_2, as in a tight channel where d=0. On the other hand, increasing *d* between the two enzymes could cause [P] to become more limiting to the reaction catalyzed by E_2. If substrates and products occur at low (micro- or nanomolar) concentrations, or if enzyme k_{cat} values are high, or if E_1 and E_2 were immobilized far from each other, the gradient in [P] may not be large enough to drive diffusion from E_1 to E_2 at sufficiently

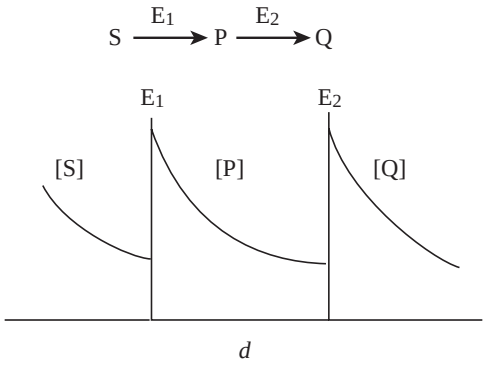

Fig. 3. Hypothetical plot of substrate (S) and product (P and Q) concentrations as a function of distance, *d*, from two sequential pathway enzymes, E_1 and E_2. In this sequence, S is converted to P and then to Q, in reactions catalyzed by E_1 and E_2, respectively. Curves denoting changes in [S], [P] and [Q] as a function of *d* are purely hypothetical.

high rates to keep the reaction catalyzed by E_2 from becoming diffusion-limited.

Evaluating what might make intuitive sense in quantitative terms is not so easy. For example, it took fairly elaborate calculations before Fell (1980) arrived at the conclusion that, despite the inhomogeneous distribution of adenylate cyclase and phosphodiesterase, intracellular concentration gradients in cyclic AMP should tend to be modest. A more general outcome of this approach is that metabolite diffusion rates over typical inter-enzyme distances are much faster than rates of enzyme turnover *in vivo*, and so metabolite concentration gradients over typical inter-enzyme distances would be expected to be negligible (Fell, 1991). Conducting calculations based on reaction–diffusion theory, Westerhoff and Welch (1992) arrived at the conclusion that the activity of the glycolytic enzymes in yeast cells would not be expected to be diffusion limited, given the concentrations of glycolytic enzymes, their k_{cat} values, cell dimensions and glycolytic flux rates. However, diffusion may become limiting to catalysis when pathway enzymes operate at high rates, when diffusion distances are large (because cells are large and/or because enzyme concentrations are low), or when product/substrate concentrations are low (Welch and Easterby, 1994).

A number of familiar reactions in muscle energy metabolism are now examined within this framework.

Creatine kinase and ATP turnover in aerobic muscles

In muscles that sustain high operating frequencies for extended durations, mitochondrial oxidative phosphorylation is responsible for most of the resynthesis of the ATP hydrolyzed by cellular ATPases. Because of the *distances* separating mitochondrial ATP synthase and actomyosin ATPase activities, as well as the *rates* at which ATP turnover occurs, it is here where we encounter one of the exceptions to the generalization that intracellular metabolite gradients are insignificant to metabolism. During exercise, there is a need for high rates of diffusive flux of ADP from myofibrils, where actomyosin ATPase catalyses the hydrolysis of ATP to ADP + P_i, to mitochondria, where ATP is resynthesized. ADP is both a substrate and a regulator of mitochondrial ATP synthesis. The free, cytoplasmic ADP concentration in muscles is so low that, at high rates of ATP turnover, it has been calculated (Jacobus, 1985) that the intracellular gradient in [ADP] could not be large enough to account for the required flux from myofibrils to mitochondria. It has been found that in vertebrate skeletal and cardiac muscles, about half of the CK occurs in the sarcoplasm and the other half occurs, as specific isoforms, bound to mitochondria and myofibrils. The problem of ADP-diffusion limitation of oxidative phosphorylation is solved through the diffusion of creatine from myofibrils to mitochondria, where, *via* the mitochondrial CK reaction, creatine accepts a phosphate group from ATP and diffuses back to the myofibrils as CrP. At the myofibrils, ATP is hydrolyzed by actomyosin ATPase and the ADP produced is rephosphorylated at the expense of CrP in a reaction catalyzed by myofibrillar CK. The essence of this cycle, called a ‘shuttle’ by some, was described by Meyer et al. (1984) and is the subject of the paper by Dzeja (2003).

A full account of the role of CK in energy transport in muscles is beyond the intended scope of this paper; however, a number of features of the process are relevant to the issues raised here. In their insightful analysis, Meyer et al. (1984) argued that the transport functions of creatine and CrP are simply consequences of the near-equilibrium nature of the CK reaction. A decade later, McFarland et al. (1994) used a spin-transfer NMR method to measure forward and reverse CK flux rates in soleus, a slow-twitch, oxidative muscle. It was found that over a tenfold range of ATP turnover rates, forward and reverse flux rates remain equal and the behaviour of CK *in vivo* is reconcilable with the kinetic properties of the enzyme *in vitro*. These results have been interpreted as providing further support for the near-equilibrium model for the ‘spatial buffering’ role of CK and the idea that solution biochemistry provides a sufficient explanation for high-energy phosphate transport in muscles. Evidence for channeling of ATP between the adenine nucleotide translocase and mitochondrial CK (Moreadith and Jacobus, 1982; Wallimann, 1996), between myofibrillar CPK and actomyosin ATPase (Arrio-Dupont, 1988), the apparent inaccessibility of a fraction of the creatine/creatine phosphate pool to CK (Hochachka and Mossey, 1998; Trump et al., 2001) and, most recently, detection of compartmentalized displacement from equilibrium of mitochondrial and myofibrillar CPKs in hearts (Joubert et al., 2002), have led others (e.g. Wallimann, 1996) to question the near-equilibrium explanation of Meyer et al. (1984). To some extent, it seems possible to reconcile these datasets and views by recognizing that soleus muscles possess much lower mitochondrial volume densities than hearts. The data obtained by McFarland et al. (1994) are therefore consistent with Meyer et al. (1984), given what is observable using ^{31}P-NMR in this system. In hearts with greater mitochondrial volume density, CK net fluxes occurring in opposite directions are observable at the mitochondria and the

myofibrils, where compartmentalized or channeled reactions occur, while equal and reversible fluxes are also observed in the cytosol (Joubert et al., 2002). Thus, intracellular high energy transport in highly aerobic muscles is made possible by channeled reactions in compartments, as well as a near-equilibrium reaction in solution (Fig. 4).

Glycolytic enzymes, their substrates and products

If, for the sake of argument, a uniform distribution of glycolytic enzymes is assumed to occur in muscles, their concentrations would range from approximately 30 to >1000 μmol l^{-1} (Srivastava and Bernhard, 1986; Albe et al., 1990; Betts and Srivastava, 1991). When enzyme concentrations are compared with their substrate and product concentrations, we find that in some cases, [S] and [P] occur in the millimolar range, exceeding [E] by 2–3 orders of magnitude. For example, phosphoglucoisomerase occurs at a concentration of approximately 33 μmol l^{-1} (Albe et al., 1990), while its substrates, G6P and F6P (glucose 6-phosphate and fructose 6-phosphate, respectively), are held close to equilibrium at millimolar concentrations (e.g. Kashiwaya et al., 1994; Staples and Suarez, 1997) in the reaction, G6P↔F6P. Although there is evidence of hexokinase binding to porin on the surfaces of mitochondria (Adams et al., 1991; de Cerqueira Cesar and Wilson, 1998; Wilson, 2003) and PFK binding to actin (Liou and Anderson, 1980; Roberts and Somero, 1987), hexose phosphate flux from hexokinase, through phosphoglucose isomerase (PGI), to PFK does not appear to occur *via* a channeled mechanism (Srivastava and Bernhard, 1986). Thus, the net flux rate at PGI, v, is equal to the steady-state rate of glycolysis and represents the difference between forward and reverse flux rates. Given the kinetic properties of the enzyme, v is defined by the Haldane equation (Haldane, 1930; Veech et al., 1969) as:

$$v = \frac{[Vf \times G6P/Kmf] - Vf \times [Kmr/(Keq \times Kmf)] \times F6P/Kmr}{1 + G6P/Kmf + F6P/Kmr}, \quad (4)$$

where Vf and Vr, $K_m f$ and $K_m r$, represent V_{max} and K_m values in the forward and reverse directions, respectively, K_{eq} is the equilibrium constant for the reaction, and G6P and F6P are the concentrations of substrate and product, respectively. PGI therefore represents an example of how a 'solution biochemistry' paradigm provides a sufficient explanation for the behaviour of a near-equilibrium glycolytic reaction in muscle during exercise (Staples and Suarez, 1997). In flying honeybees, the steady-state flux rate through glycolysis is approximately 30 μmoles g^{-1} min^{-1}, while the capacity for flux at the PGI step is more than 30-fold higher. The high capacity for flux is not in excess, but actually represents the activity required to make possible the net forward (glycolytic) flux rate, while maintaining near-equilibrium (Staples and Suarez, 1997).

In contrast, the steady-state concentrations of pathway substrates and products of a number of reactions further downstream in glycolysis [e.g. aldolase (ALD), triosephosphate isomerase (TPI), glycerol 3-phosphate dehydrogenase (G3PDH), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK)] occur in the micromolar range (Veech et al., 1969; Connett, 1985; Kashiwaya et al., 1994). Under these conditions, a large fraction of pathway intermediates may be bound to enzyme active sites (Veech et al., 1969; Connett, 1985) and, at high flux rates, the enzymes could be diffusion-limited if they were uniformly distributed within the sarcoplasmic space. In work to be described in greater detail and, no doubt, updated by Sullivan (2003), Wojtas et al. (1997) made transgenic *Drosophila* with G3PDH lacking a C-terminal tripeptide required for binding to *Z*-discs and *M*-lines. Failure of G3PDH to localize also resulted in failure of GAPDH and ALD to colocalize at these sites and, as a result, the flies could not fly. Although metabolic data are lacking, these results have been interpreted as being consistent with the need for these enzymes to be held in close proximity to each other. It is possible that, given the low, near-equilibrium concentrations of substrates and products, close proximity of active sites is required to overcome diffusion limitations that may occur at the high metabolic rates (Lehmann et al., 2000) required to sustain flight. Such an explanation may be intuitively

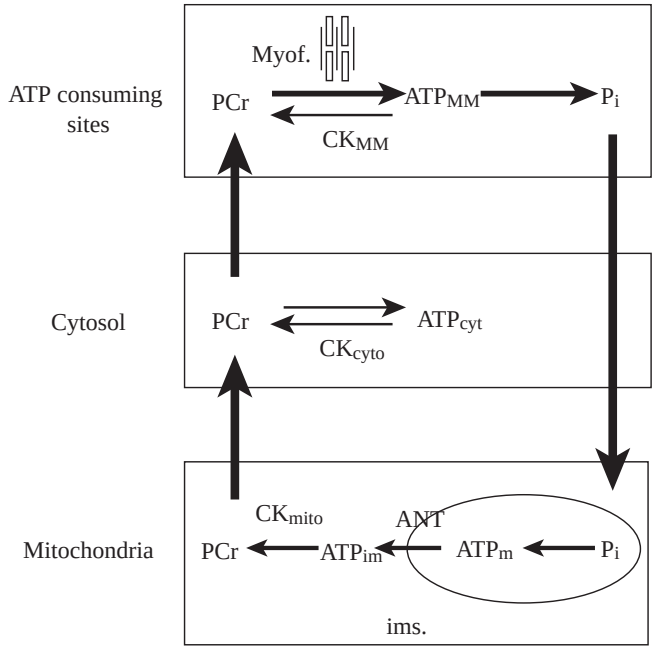

Fig. 4. Creatine kinase fluxes in working hearts, as resolved by ^{31}P-NMR spectroscopy (redrawn and modified from Joubert et al., 2002). The diagram shows metabolites and fluxes occurring in three compartments: ATP-consuming sites (myofibrils only shown for simplicity), cytosol and mitochondria. In the study of Joubert et al. (2002), unidirectional fluxes were detected at the myofibrils and mitochondria, while CK fluxes in both directions are detected in the cytosol. Myof., myofibrils; CK_{MM}, myofibrillar creatine kinase; CK_{cyto}, cytoplasmic creatine kinase; CK_{mito}, mitochondrial creatine kinase; ANT, adenine nucleotide translocase; ims., intermembrane space; ATP_{MM}, ATP at the myofibrils; ATP_{cyt}, cytoplasmic ATP; ATP_{im}, ATP in the ims.; ATP_m, ATP in the matrix space.

appealing, but it leaves open the question of what actual mechanisms are involved. The enzymes may bind specifically to each other to form stable complexes or, they may form weak, transient associations. If channeling does occur, it would be interesting to know if it operates *via* a perfect or leaky mechanism. Finally, given the observation that the D_{cyt} of some metabolic enzymes changes with metabolic state (Verkman, 2002), it is conceivable that the degree of channeling or the relative leakiness of channeled reactions may change with transitions between rest and exercise.

Does convection help or hinder muscle metabolism?

Because many cell types are known to expend energy to promote cytoplasmic streaming, (e.g. Sugi, 2003) it is tempting to assume that fluid convection is a universally desirable phenomenon. When a muscle fiber shortens and lengthens while maintaining constant volume, its cross-sectional area would be expected to increase and then decline in cyclic fashion. While this occurs, the distances between actin and myosin filaments would remain constant, but as fiber cross-sectional area increases and declines, water molecules might undergo cycles of radial displacement and replacement. The exact nature of these water movements may be difficult to predict at the small spatial scales, low Reynolds numbers, muscle strains and operating frequencies involved. However, this raises the possibility that, in addition to their random, diffusive walks through the sarcoplasm, enzyme molecules may be subjected to convective movement. That convection may be unimportant in muscles might be suggested by that lack of effect of muscle contractions on myoglobin diffusion (Papadopoulos et al., 1995). Nevertheless, given the idea that close proximity might be necessary between enzymes operating at high rates and at low substrate concentrations, the disruption of such associations by convective movement may provide another explanation for why anchoring to intracellular sites, as well as to other enzymes, may be required for flies to fly.

Interspecific variation is interesting and important

Muscles differ in structure, mechanical function and biochemical properties within individuals and species, as well as across species. Such variation provides the opportunity to explore the extent to which mechanisms, established using only a few model systems and considered to be 'fundamental,' apply to other species over a range of lifestyles, body sizes, and metabolic rates. It also provides an opportunity to test hypotheses concerning the true adaptive significance of proposed mechanisms. Consider, for example, the apparent absence of a mitochondrial form of arginine kinase in locust flight muscles (Schneider et al., 1989). If creatine and CrP have assumed such important roles in energy transport in vertebrate muscles, why should arginine and arginine phosphate not play such roles in insect flight muscles, considering their even higher rates of aerobic ATP turnover? Could the mitochondrial volume densities in insect flight muscles be so high that, despite high rates of ATP turnover, diffusion distances are small enough to allow ADP to travel from the myofibrils to serve as the mitochondrial phosphate acceptor, without causing diffusional limitations? Alternatively, are arginine and arginine phosphate involved in the spatial buffering of ATP concentrations by way of the near-equilibrium mechanism proposed by Meyer et al. (1984)? Consider also hexokinase binding to porin on mitochondrial surfaces and the channeling of ATP from the adenine nucleotide translocase to this enzyme, a topic covered by Wilson (2003). It has been proposed that this may serve as a mechanism by which glucose phosphorylation is coordinated with mitochondrial oxidative phosphorylation in aerobic cells. In contrast, *Drosophila* hexokinases do not contain the appropriate hydrophobic sequences required for binding to porin (Duvernell and Eanes, 2000), and preliminary experiments in my laboratory (J. Staples and R. K. Suarez, unpublished observations) have failed to reveal evidence of hexokinase binding to mitochondria in honeybee *Apis mellifera* flight muscles, a system in which most of the carbon fueling mitochondrial oxidative metabolism must go through the hexokinase reaction! These examples serve to illustrate how conclusions derived from the results of experiments using more conventional model systems can be further evaluated by application of the comparative method. Far from being trivial exercises, such comparative studies provide a means by which the fundamental nature of biochemically interesting phenomena can be assessed.

Reconciling the old and the new

It seems just as naïve to suppose that all metabolic reactions occur *via* channeling as it is to think that all of cellular metabolism occurs in a homogeneous soup. It should be hardly surprising to biologists, given the diversity and complexity of animal life, that a broad spectrum of possibilities has been explored during evolution and employed by animals to various degrees and in various combinations that might depend on species, muscle type and physiological state. This degree of complexity, if anything, calls for greater rigor and a reluctance to accept sweeping generalizations at face value. It has been said, and no doubt in more than just a few biochemical conferences, that because metabolic channeling is so ubiquitous, V_{max} and K_m values measured *in vitro* are meaningless or, at best, of questionable relevance. However, some approaches in metabolic control analysis (an approach that has revolutionized studies of metabolic regulation) make extensive use of kinetic parameters estimated *in vitro* (Fell, 1997). For example, the most comprehensive study of the control of glycolysis of which this author is aware makes use of the Haldane equation and enzyme kinetic parameters to estimate the control coefficients of each of the glycolytic reactions in perfused rat hearts (Kashiwaya et al., 1994). Recently, a computer model that makes use of the *in vitro* kinetic properties of glycolytic enzymes (Lambeth and Kushmerick, 2002) allowed the prediction of glycolytic flux rates in ischemic muscle (Lambeth et al., 2002). It would appear that the wholesale abandonment of 'old' concepts and

approaches in favour of blanket generalizations, though at times fashionable, is not the path to progress. NMR spectroscopy, Haldane, metabolic control theory and molecular genetics must be reconciled if we are to come to a more realistic understanding of muscle structure and metabolism.

It is with eternal gratitude that I dedicate this paper to the memory of my mentor, colleague and dear friend Peter Hochachka. Thanks are due Martin Kushmerick, John Gosline, Charles Darveau, John Wilson and David Sullivan for helpful discussions, and the US National Science Foundation (IBN 0075817) for research support.

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