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Review

Intracellular convection, homeostasis and metabolic regulation

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Summary

Two views currently dominate experimental approaches to metabolic regulation. The first, let us call it Model 1, assumes that cells behave like a watery bag of enzymes. The alternative Model 2, however, assumes that 3-dimensional order and structure constrain metabolite behavior. A major problem in cell metabolism is determining why essentially all metabolite concentrations are remarkably stable (homeostatic) over large changes in pathway fluxes – for convenience, this is termed the [s] stability paradox. During large-scale transitions from maintenance metabolic rates to maximally activated work, contrasting demands of intracellular homeostasis *versus* metabolic regulation obviously arise. Data accumulated over the last 3–4 decades now make it clear that the demands of homeostasis prevail: during rest–work transitions, metabolites such as ATP and O_2 are notably and rigorously homeostatic; other intermediates usually do not vary by more than 0.5- to threefold over the resting condition. This impressive homeostasis is maintained despite changes in pathway fluxes that can exceed two orders of magnitude. Classical or Model 1 approaches to this problem can explain metabolite homeostasis, but the mechanisms for each metabolite, each enzyme locus, are necessarily specific. Thus Model 1 approaches basically do not provide a global explanation for the [s] stability paradox. Model 2 takes a different tack and assumes that an intracellular convection system acts as an over-riding 'assist' mechanism for facilitating enzyme–substrate encounter. Model 2 postulates that intracellular movement and convection are powered by macromolecular motors (unconventional myosins, dyneins, kinesin) running on actin or tubulin tracks. For fast and slow muscle fibers, microfilaments are concentrated near the periphery (where convection may be most important), but also extend throughout the actomyosin contractile apparatus both in horizontal and vertical dimensions. To this point in the development of the field, Model 1 and Model 2 approaches have operated as 'two solitudes', each considering the other incompatible with its own experimental *modus operandi*. In order to finally assemble a model that can sensibly explain a realistic working range of metabolic systems, opening of channels of communication between the above two very differing views of metabolic regulation would seem to be the requirement for the future.

Key words: metabolic regulation, homeostasis, intracellular, diffusion, intracellular perfusion, oxygen delivery, oxygen regulation.

Contrasting demands of homeostasis and tissue work

In its historic definition, the term homeostasis refers to the constancy of the internal milieu in the face of external perturbations; the latter in principle may be caused by extracellular factors, or in the case we shall consider, by change in intracellular biological function. Of all the tissues in the vertebrate body, skeletal muscle displays the special quality of being able to routinely sustain very large changes in work and metabolic rates. Compared to the 1.5- to 2-fold differences in metabolic rates between resting and activated states, which is common to many tissues (liver and brain, to mention two), skeletal muscles in most animals must be able to sustain up to, or even more than, 100-fold changes in ATP turnover rates. Amongst vertebrate endotherms, the highest muscle metabolic rate (in the range of 600 μmol ATP g^{-1} min^{-1}) appears to be that of hummingbird breast muscle during hovering flight, which is a rate over 500 times muscle RMR (Suarez, 1992; Suarez et al., 1990, 1991). During muscle ischemia, hypoxemia or hypoxia, the metabolism of muscle, like that of many other tissues under conditions of oxygen lack, may need to sustain a suppression of metabolism even below resting rates (Hochachka and Guppy, 1987), thus extending even further the enormous range between the lowest and highest sustainable ATP turnover rates of this remarkable tissue.

Current popular interpretations of such large scale differences in steady state energy turnover are regulated assume cybernetic feedback control circuitry. The standard

theory is summarized in Fig. 1 (see Balaban, 1990; Chance et al., 1986; Connett, 1988; Connett et al., 1985; Connet and Honig, 1989; From et al., 1990; Kushmerick et al., 1992; Rumsey et al., 1990). Following the arrival of activation signals at the muscle cell, an increase in ATP demand 'turns on' cell ATPases, whose catalytic function leads to increased product (ADP, P_i, H^+) concentrations; the latter then serve as substrates and as positive feedback signals for accelerating ATP supply pathways (Fig. 1). Metabolites such as ADP and P_i are thought to be pivotal in mitochondrial metabolic control, but powerful activation of cell work also demands a proportional activation of catalytic function at essentially every enzyme step involved in the ATP supply and demand pathways. Hence, if substrate, product and modulator concentration changes are to be the main mediators of large (100-fold or more) changes in ATP turnover rate, one would anticipate equally large perturbations in pool sizes of the numerous intermediates. This would be especially true for regulation processes based on Michaelis–Menten kinetics, where the kinetic order cannot exceed 1 [Atkinson, 1977, 1990; i.e. percentage change in catalytic rate (ATP turnover rate) cannot exceed the percentage change in substrate concentration driving the metabolic rate change (Hochachka and Matheson, 1992; Hochachka, 1994)]. Whereas 'homeostasis' demands 'constancy of the internal milieu', muscle work would thus appear to require drastic changes in intracellular conditions, the degree of perturbation being somehow related to the intensity of work. The problem (and paradox) is how the conflicting demands of homeostasis *versus* metabolic regulation are resolved in muscle during different work and metabolic states; i.e. how muscles sustain both metabolic homeostasis and metabolic regulation.

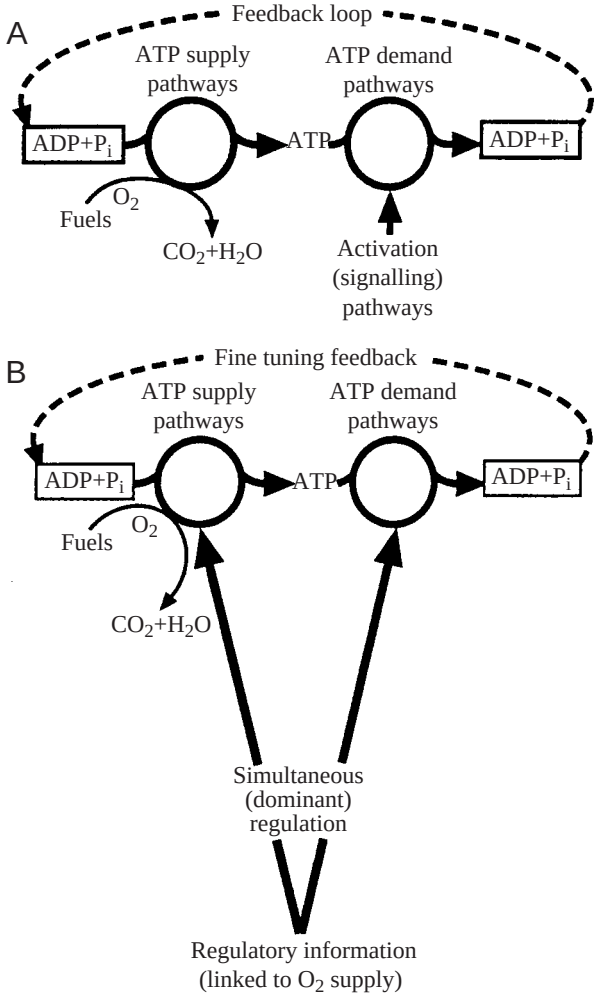

Fig. 1. (A) Summary diagram of the standard model of metabolic regulation; in this conceptualization, accelerating tissue ATP utilization leads to increasing concentrations of ADP and P_i, which serve as substrates for oxidative phosphorylation, to activate ATP production. (B) Summary of an alternative model of metabolic regulation in which ATP demand and ATP supply pathways are simultaneously activated during large-scale change in tissue work rates. See text for further details.

Two views or approaches dominate the metabolic regulation field

Over the last 3–4 decades, two general frameworks (we shall term them Models I and II) accounting for metabolic regulation have dominated thinking in the field. These two views can be nicely illustrated by considering the vertebrate phosphagen system. Model I assumes: (i) that the total acid-extractable pool of Cr + PCr (termed tCr) occurs in aqueous solution and is fully accessible to creatine phosphokinase, CPK, (ii) that solution chemistry rules apply globally in muscle cells *in vivo*, and (iii) that the main CPK-phosphagen function is to 'buffer' ATP concentrations during large-scale changes in muscle work and in ATP turnover rates. Model II hypotheses consider: (i) that the structural organization of phosphagen-containing cells physically constrains tCr, (ii) that solution chemistry rules may apply *in vivo* mainly to localized PCr/Cr pools, and (iii) that intracellularly localized CPK isoforms *in vivo* create complex and possibly directional pathways of PCr and Cr metabolism – forming so-called creatine shuttles in metabolism. Model I considers the cell essentially as a bag of enzymes in which simple solution chemistry rules apply; Model II sees the cell as a highly structured system with intracellular ultrastructure incorporating constraints on metabolic processes and, in the extreme, imposing three-dimensional order on metabolic function. The polarization illustrated by these two views extends throughout the metabolic regulation field and has caused the field to progress along two surprisingly independent paths with minimal communication between them. Both views, however, must accommodate the empirical observations of pathway fluxes and pathway intermediates under different metabolic states.

The Model I approach

Regulatory properties of working muscles

It is a rule of thumb in biology that many physiological and molecular functions are the sum of individual processes linked

in sequence; in isolation many such individual processes have no clear functions at all. For metabolic pathways, integrated function often is evaluated by comparing changes in flux through the pathway *per se* with changes in concentrations of substrates and products of individual enzyme reactions within the pathway. Such approaches indicated very early on that enzymes in multistep pathways are surprisingly well integrated. Examples of this are shown for ^{31}P magnetic resonance spectroscopy (MRS)-visible phosphate metabolites for human muscle work, in essence monitoring the path of ^{31}P during ATP turnover. These data show that [PCr] and [Pi] both change with muscle work, that the change is an accurate reflection of the change in ATP demand, and that the quantitative response differs in slow *versus* fast twitch fibers. It is less marked in the former than the latter, and these metabolites are even less (actually immeasurably) perturbed during work transitions in the heart. What is more, ATP concentrations are perfectly protected through these kinds of work transitions in all of three (slow, fast and cardiac) muscle types. Similar data exist for the path of glycolysis in rat and fish fast twitch fibers, and in rat heart. Again, even if change in flux demanded by change in work perturbs the concentrations of pathway intermediates, what is most striking is how small these changes are compared to the huge changes in pathway flux. Even in extreme cases, such as in very high capacity metabolic pathways in insect flight muscles or in the electric organ of electric fishes, >100-fold flux changes in pathways of ATP demand and supply can be achieved with only minor perturbation in concentrations of pathway intermediates.

To explain this precision and integration of linked sequences of enzyme function, several regulatory models are currently being evaluated by workers in this field (Hochachka et al., 1998). These include: (i) simple feedback and mass action controls (the standard model above), (ii) allosteric controls, (iii) models involving the regulation of e_o (the concentration of functional catalytic sites) in various ways, such as by alteration in protein interactions (as in actomyosin ATPase), change in phosphorylation state (as in pyruvate dehydrogenase), change in redox state (as in V-type ATPases), by Ca^{2+} activation, or translocation from inactive to an active intracellular location (as in glucose transporters), and (iv) various versions of metabolic control analysis originally introduced over a decade ago (these make minimal assumptions at the level of enzyme mechanism). The diversity of mechanisms and models of enzyme regulation arise in part from the differing requirements at different enzyme loci in metabolism.

Enzyme catalytic and regulatory properties determine control requirements for specific metabolic reactions

Many, and perhaps most, enzymes in metabolic pathways obey Michaelis–Menten kinetics and operate under near-equilibrium conditions (at equilibrium, of course, forward and reverse fluxes for such enzyme reactions are the same and there can be no net forward or reverse flux). During pathway and enzyme activation (Staples and Suarez, 1997), net forward flux for such enzymes is achieved by modest adjustments in substrate(s)/product(s) concentration ratios. Several requirements arise for such enzymes *in vivo*. First of all, since the chemical potential driving the net forward reaction is usually modest, large amounts of enzyme are the rule in order to be able to match the flux rates required *in vivo*. This is achieved by high enzyme content or by high k_{cat} (turnover number per active site on the enzyme), or by both mechanisms at once. Traditionally, for most metabolic biochemists, this is the explanation for 'near-equilibrium' enzymes occurring at relatively enormous concentrations – the higher the enzyme concentration, the closer to equilibrium is *in vivo* function – and for 'near-equilibrium' enzymes being catalytically especially efficient. Triose phosphate isomerase (TPI) is one such example. In tissues with a high (aerobic or anaerobic) glycolytic potential (such as various fast twitch muscles) the enzyme occurs at almost mmol l^{-1} concentrations and its high k_{cat} means that its *in vivo* activity (in terms of μmol substrate converted to product g^{-1} min^{-1}) is enormous. In fact, studies carried out over two decades ago (see Hochachka, 1980, 1994; Fersht, 1985) showed that selection for high efficiency has pushed this enzyme towards a state of catalytic 'perfection': any further improvement in the enzyme's efficiency would not be expressed in higher reaction rates because it would be limited by diffusion-based enzyme–substrate encounter.

Most metabolic pathways also contain allosteric enzymes that function under quite different conditions and are much more subject to regulation. Allosteric regulation (Fersht, 1985; Hochachka and Somero, 1984) is based on positive or negative modulators binding at sites other than the active site (hence the term 'allosteric' rather than isosteric, which would apply to modulators competing with substrate at the active site). Phosphofructokinase (PFK) in glycolysis is a quintessential example of an allosteric enzyme. PFK *in vivo* operates far from equilibrium, tending to work largely in the forward direction, catalyzing the reaction

$$F6P + ATP \rightarrow ADP + F1,6P\ .$$

The enzyme is product-activated by both ADP and fructose-1,6-phosphate (F1,6P), by fructose-2,6-phosphate (F2,6P), AMP, ammonia and high pH. Fructose-6-phosphate (F6P) saturation curves are sigmoidal with an interaction coefficient, *n*, of 2 or more. This means that when one F6P is bound the affinity of the next site for F6P rises; in this sense, F6P is both a substrate and an activator of the enzyme acting upon it. Most modulators affect PFK by shifts in substrate affinities, rather than in $\dot{V}_{max}$, except for the cosubstrate ATP: at high concentrations ATP becomes a substrate inhibitor of the reaction. Taken together, these regulators add up to a pattern in which energy-rich conditions downregulate PFK activity, while energy-depleted conditions upregulate PFK activity. These properties mean that the chemical potential driving the forward reaction tends to be very high and high fluxes can therefore be sustained with lower enzyme activities (i.e. lower enzyme concentrations are required and the lower k_{cat} values are quite tolerable).

Still other enzymes are regulated essentially in on–off fashion. Pyruvate dehydrogenase (PDH), for example, is regulated by phosphorylation–dephosphorylation mechanisms (by protein kinases and protein phosphatases, respectively). The ATP-dependent PDH kinase-catalyzed reaction converts PDH to the low-activity (off) form, which is favored under energy saturated conditions, while a phosphatase hydrolyzes this bond, releasing P_i and converting PDH back to its high-activity (on) form. This may be viewed as a kind of coarse control system; fine control of PDH catalytic activity is achieved by NADH and acetylCoA product inhibition. As in the case of PFK, PDH functions far from equilibrium and the chemical potential driving the forward reaction is high. This means that the same maximum *in vivo* flux capacities can be matched by lower enzyme concentrations and lower catalytic efficiencies than in the case of near-equilibrium enzymes such as TPI.

Similar phosphorylation-based on–off control types of enzymes activity were first discovered for glycogen phosphorylase in the 1960s and have been shown for numerous other enzymes. Although protein kinase and phosphatase couplets are the most common, they are not the only means by which enzymes can be held in either on or off states. Two other mechanisms to briefly mention are those based on protein–protein interactions and on redox change in –SH residues. Actomyosin ATPase is an example of the former (Grabarek et al., 1992) and V-type ATPAse of the latter (Harvey and Wieczorek, 1997). At rest actomyosin ATPase in skeletal muscle is catalytically inert, held that way by troponin c. Ca^{2+} activation (during excitation–contraction or EC coupling) relieves troponin c binding and unleashes this enzyme's huge catalytic activity. Low- and high-activity forms of V-type ATPases are similarly regulated by hypoxia or other parameters that change local redox conditions (reducing the S–S bridge to –SH). Because the concentration of catalytically active sites (e_o) is effectively low in the off state and high in the on state, this category of mechanisms is referred to as e_o regulation of enzyme activity (Hochachka and Matheson, 1992). The exceptionally huge metabolic flare up associated with electric organ discharge in electric fishes may supply a particularly clear example of this kind of regulatory mechanism (Blum et al., 1990, 1991).

Homeostasis of pathway intermediates

The largest problem (and paradox) facing metabolic regulation theory

Given this diversity in the nature of enzymes that *in vivo* are linked together to form single metabolic or physiological functions, it is all the more perplexing to find, and challenging to account for, the empirical observation that enzymes linked in linear series to form metabolic pathways are so exquisitely integrated that large changes in pathway flux are sustained with minimal perturbation of pathway substrates and products. [ATP] is almost perfectly homeostatic under most conditions (except under very extreme O_2-limited or fatigue conditions) and other intermediates in pathways of ATP supply or demand are stabilized within less rigorously controlled concentration ranges (where these changes may reflect change in ATP turnover rates, but clearly cannot cause them. A cursory count shows that the percentage changes in concentrations of >60 substrates and intermediates (in glucose, fat and amino acid catabolic pathways) quantified to date are far less than the percentage changes in flux rates with which they correlate. This is observed over and over again, for low-capacity and high-capacity pathways. Although on first analysis oxygen appeared to be the only metabolite that was an exception, even this crucial metabolite turns out to be impressively homeostatic.

Aerobic metabolic rate and oxygen delivery ($\dot{Q}_{O_2}$) are closely related

There is a huge literature on how O_2 functions both as a substrate and as a potential regulator of tissue metabolism over varying times of exposure and we shall not review this comprehensively at this time. There are both physiological and biochemical aspects to controlling the relationship between O_2 delivery and consumption. As energy demand changes, physiological mechanisms must be harnessed for appropriate perfusion changes. Multiple metabolite signals (adenosine, K^+, H^+, endothelins, nitric oxide) are utilized for coordinating perfusion with cell-level energy demands. Nitric oxide (NO) has received particular attention over the last decade. NO is formed from arginine in a reaction catalyzed by nitric oxide synthase (NOS), which in mammals occurs as three different isoforms (Förstermann and Hartmut, 1999). NOS I or ncNOS was originally discovered in neurons, NOS II or iNOS in cytokine-induced macrophages, and NOS III or ecNOS in endothelial cells. This field of research is far too large to explore in detail here. Suffice to point out that NO released by NOS catalytic activity serves in perfusion regulation by direct vasodilation, and indirectly through hemoglobin (Hb) binding. The latter mechanism is only now being worked out in detail, but it already appears that NO binding to Hb occurs at the lungs while its release is favored at the tissues; the lower the oxygen tension, the greater the need for NO-mediated vasodilation, and the greater the NO release from Hb (Gow et al., 1999). These kinds of studies go a long way towards explaining why numerous studies have found essentially a 1:1 relationship between $\dot{Q}_{O_2}$ and tissue (especially muscle) work. For example, recent studies using dog gastrocnemius muscle (Arthur et al., 1992), found such a relationship over an 18-fold change in ATP turnover rate. Later, Hogan et al. (1992) used the same preparation to analyze subtle submaximal work changes; these transitions were sustained with no change at all in concentrations of PCr, ATP and other metabolites. Yet through these transitions a 1:1 relationship between $\dot{V}_{O_2}$ and $\dot{Q}_{O_2}$ was maintained, and these results are similar to many other data from other laboratories on different tissues and organs. This leads to the conclusion that this is the only metabolite signal so far identified that varies 1:1 with work over realistic biological rate changes. That is why we and many others in the field accept that O_2 plays a key role in regulating up- or down-

change in ATP turnover. But how is the O_2 signal transduced within the cell?

Oxygen signal transduction in working muscle does not rely on change in intracellular [O_2]

Interestingly, the answer to the question posed above remains unclear. So far the only mechanisms proposed from traditional studies in this area assume simple diffusion paths from capillaries and calculate smooth diffusion gradients within the cell ending in mitochondrial O_2 sinks. However, this approach has been less than satisfactory for, to unravel the puzzle of how O_2 delivery translates into effects on metabolism within the cell, we require hard data on intracellular O_2 concentration. The difficulty is that for most tissues this key parameter remains elusive and unknown; only in muscle is the situation more favorable. In this tissue, myoglobin (Mb) is a direct intracellular detector of [O_2]. Mb is a relatively small, monomeric respiratory pigment occurring in heart and mitochondria-rich skeletal muscles at concentrations of less than 0.5 mmol l^{-1}; in muscles of marine mammals such as seals, Mb concentrations reach into the 4–5 mmol l^{-1} range. Gene knockout experiments (Garry et al., 1999; Goedeke et al., 1999) show that even if mice can survive without Mb they can do so only by activating compensating mechanisms such as increasing capillary densities and blood O_2 carrying capacity. It is therefore usually assumed that Mb is functionally important under the usual physiological conditions. At 37°C, O_2 solubility in physiological solutions is approximately 1 μmol l^{-1} $torr^{-1}$ (1 torr≈133.3 Pa). Because the reaction $Mb+O_2 \leftrightarrow MbO_2$ is always in equilibrium, with a P_{50} of 3 torr (K_d of approximately 3 μmol l^{-1}), whenever [O_2] is less than saturating for Mb, $\%MbO_2$ directly estimates intracellular [O_2].

Earlier attempts to make such estimates with working muscle preparations almost exclusively relied upon near infrared spectroscopy. More recently, MRS is being used to take advantage of a histidine-H being 1H MRS 'visible' in deoxyMb but being MRS 'invisible' in oxyMb (Richardson et al., 1996). For the first time, this new technology supplies workers in the field with a noninvasive window on the oxygenation state of muscles in different work and metabolic states, at least for muscles with a high enough [Mb] to be 1H MRS 'visible'. When first applied to both working human skeletal muscles (Richardson et al., 1996) and to heart (Jelicks and Wittenberg, 1995) the same instructive results were found: essentially stable $\%MbO_2$ through large changes in work rate. In such studies, as soon as a work load is imposed (even in very low intensity exercise, such as unloaded pedaling), $\%MbO_2$ quickly establishes a new steady state, usually between 40% and 70% saturation, both as a function of time and as a function of tissue work intensity. Along with gold labeling studies showing a random Mb distribution in rat heart and skeletal muscles (S. Shinn and P. W. Hochachka, unpublished observations), the MRS data imply that $\%MbO_2$ and intracellular [O_2] both remain relatively constant up to the maximum sustainable aerobic metabolic rate of the tissue. Just as CPK serves to 'buffer' ATP concentrations during changes in muscle work so Mb apparently serves to 'buffer' intracellular [O_2] in different metabolic states.

Parenthetically, it should be acknowledged that the volume of interest in such MRS studies is large and the MRS data necessarily are averages obtained from large numbers of fibers. Human muscles, like muscles in other mammals, are formed from mixtures of fiber types and as work intensity rises for a given muscle mass, there may be changes in recruitment and in the percentage contribution of different fiber types. This problem does not arise in studies of heart muscle, which is biochemically rather homogenous. While Richardson et al. (1996) apparently avoided this artifact, this does not seem to be the case in the study by Mole et al. (1999) on an unknown mix of fibers in human calf muscle. Evidence of the problem initially arises from their ^{31}P MRS data, which showed an expected linear decrease in [PCr] as work increased; at maximum aerobic work, [PCr] changed maximally by approximately threefold. Since the same [PCr] change occurs when gastrocnemius work rate reaches only 40% of sustained aerobic maximum, but much smaller changes in [PCr] occur in (the mainly slow fibers of) soleus during the same work transition (Allen et al., 1997), it is probable that the regions of interest in Mole et al.'s study (Mole et al., 1999) may have overlapped into muscles rich in slow twitch fibers, where the change in [PCr] is less for a given level of work than in fast twitch fibers. Otherwise it would be difficult to understand why their preparation had to be pushed to its maximum work level to achieve the same %[phosphagen] shifts that Allen et al. (1997) observed at only 40% of aerobic maximum. For these reasons, the $\%MbO_2$ values recorded at different work intensities almost certainly represent different combinations of fiber types. Nevertheless, these studies found that at about 50% and 80% of sustained aerobic maximum work rate (representing huge ATP turnover rates, equivalent to about 50–80 μmol ATP g^{-1} min^{-1}), $\%MbO_2$ did not change significantly (stabilizing at approximately 65–70 $\%MbO_2$), in agreement with earlier studies; however, at the maximum work rate, a further modest desaturation to approximately 50 $\%MbO_2$ occurred, which is not in full agreement with the data of Richardson et al. (1997). Because of the mixed fiber and recruitment problems, readers should not be surprised by these modestly different results; and, at least tentatively, we consider that the small discrepancies probably arise from artifacts caused by differing metabolic states in different fiber types. Thus they do not strongly influence our main conclusion that [O_2] is largely homeostatic.

In fact, even if most workers probably would accept that Mb should function to buffer intracellular [O_2], the significance of this has not been fully appreciated. As Carl Honig pointed out in a discussion in 1987, this may be because of a too enthusiastic acceptance of traditional diffusion models assuming smooth gradients across the capillary–muscle cell threshold all the way to the mitochondrial sinks. Such models, which assume complete homogeneity and necessarily ignore the issues of fiber type and recruitment heterogeneity, are not

accepted by the Honig group. According to Honig et al. (1992), the structure of the capillary–muscle system develops steep gradients (and localized high O_2 fluxes) only at the capillary–muscle interface but very shallow gradients within the muscle cell *per se*, as indeed was found by the more recent MRS data on $\%MbO_2$ *in vivo*. That is why Hochachka and McClelland (1997) accepted the MRS data on $\%MbO_2$ at face value and emphasized that, under normoxic conditions, O_2 is perfectly homeostatic in the sense that its concentration is stable even while its flux to cytochrome oxidase can change by two or more orders of magnitude. In the examples above, the concentration of O_2 ranged between 2 and 4 μmol l^{-1} during pathway flux changes from approximately 1 to >80 μmol ATP g^{-1} min^{-1} (these high mass-specific metabolic rates are achieved because most of the cardiac output during these protocols is available for supporting the work of relatively small muscle masses).

To recapitulate, the situation arising from these new studies of oxygen and metabolic regulation can be summarized as follows. First, because of the buffering role of Mb, oxygen concentrations are low (in the P_{50} or K_d range) and intracellular $[O_2]$ gradients must be quite shallow. Second, it is emphasized by Honig et al. (1992) that the capillary–muscle contact surface area is only a fraction of the surface area of inner mitochondrial membranes and cristae; by definition this means that the highest gradients and highest O_2 fluxes are at the capillary–muscle cell threshold and that these gradients are necessarily much shallower in the cytosol. Thirdly, the low intracellular [oxygen] remains essentially stable (i.e. remains effectively buffered by the $MbO_2 \leftrightarrow Mb+O_2$ equilibrium, throughout large changes in work and metabolic rates. Nevertheless, $\dot{V}_{O_2}$ and O_2 delivery are closely related, suggesting a key role for O_2 in metabolic regulation.

Previously proposed mechanisms accounting for O_2 flux from capillaries to mitochondria

Given that it is $\dot{Q}_{O_2}$ – not intracellular $[O_2]$ – that correlates with work rate, the problem remaining is how the O_2 signal is transmitted to the machinery of cell metabolism. At this time, we admit that there is no widely accepted resolution of this problem. The traditional answer, of course, assumes oxygen diffusion down smooth concentration gradients from capillary plasma to tissue mitochondria. A second mechanism assumes accelerated intracellular O_2 transfer by Mb-facilitated diffusion in Mb-containing cells (in this case two species are diffusing to the mitochondrial targets at once: molecular O_2 and MbO_2). A third mechanism assumes that lipids (either phospholipid bilayers or triglyceride droplets) form the preferred path of O_2 diffusion because of the greater O_2 solubility in lipids than in aqueous phase. A final mechanism is that of O_2 sensing. When we first recognized this puzzling problem of O_2 transmission and realized the limitations of the classical diffusion model to explain the observations, we postulated an O_2 sensing system, presumably located in the cell membrane (or even more distally), and signal transduction pathways or mechanisms for 'telling' the cell metabolic machinery when and how potently to respond to changing availability of O_2 (Hochachka, 1994). Whereas each of the above explanations of O_2 transfer can claim to be able to account for observed O_2 fluxes under some conditions, none of the these mechanisms above are able to easily explain why the flux rates vary with O_2 delivery rather than with intracellular O_2 concentrations.

All of the above mechanisms represent so-called traditional explanations and, as briefly mentioned above they are formulated within the Model I framework of cell function: this is the view of cells as watery bags in which solution chemistry rules basically dominate the functional behavior of the system. Model II views of the cell are very different and assume that intracellular conditions are so complex that solution behavior is not necessarily the rule. The Model II view (of metabolic regulation in general and of O_2 regulation in particular) takes an entirely different tack and postulates that intracellular circulation, not diffusion, is the main means of bringing ligands and their binding sites together during upwards or downwards transitions in metabolic and tissue work rates. Let us review how this picture differs from the more classical or traditional framework discussed above.

The Model II approach

Intracellular structure needs an intracellular perfusion system

Conceptually, the major difference between the above traditional approach to metabolic regulation and Model II is the emphasis placed upon intracellular order and structure. The points of departure for the latter view are three different lines of evidence favoring intracellular perfusion but not favoring diffusion as the dominant means for regulating enzyme–substrate encounter (including cytochrome oxidase–O_2 encounter).

First and most fundamental is the structural argument: ultrastructural, histochemical and cytochemical studies do not reveal the cell as a static bag of substrates and enzymes, but rather a three-dimensional, membrane-bound microcosm housing an internal milieu filled with complex organelles, motors, membranes, cables, trabeculae and channels. Rather than a static, dead-still solution (as would be required for formal application of laws of diffusion), the internal medium is very much 'alive' in the sense that movement is the rule of thumb, movement of organelles, of particles, and of cytosol (so-called cytoplasmic streaming at rates of up to 2–3 m s^{-1}). In contrast to what might be expected of a bag of enzymes and substrates, over a half-century of research has clearly concluded that many metabolic pathways and their component enzymes are restricted to specific cell compartments, and numerous so-called soluble enzymes show intracellular binding to specific intracellular sites. Order and structure is the name of the game, as far as the literature on cell ultrastructure is concerned, and it is not a diffusion-dominated game. Take away the order and the system behaviour falls apart; sometimes function is lost completely. A good recent example of this comes from genetic studies of *Drosophila* flight muscle metabolism. While earlier studies had shown that aldolase,

glyceraldehyde 3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase colocalize mainly at *Z*-discs, Wojtas et al. (1997) used clever genetic manipulations (that influenced binding but not overall catalytic activities) to show that mislocating these enzyme activities in the cytosol rather than correctly bound to *Z*-discs would render *Drosophila* flightless – a dramatic demonstration that even if all three enzymes are expressed at high activities, their three-dimensional organization is part and parcel of *in vivo* regulated function of the pathway.

Second is the argument on macromolecular functional constraints. As we might expect from the above (and indeed find), the intracellular mobilities of enzymes and of carrier proteins such as Mb are not equivalent to those in simple aqueous solutions. For example, intracellular diffusibility estimates for Mb in the cytosol range from as low as one tenth of that found in simple solutions (Juergens et al., 1994) to values of about half that in simple solution (Wang et al., 1998). Interestingly, the latter MRS study estimated rotational diffusion, while the former study estimated translational diffusion; as indicated below, these may change independently. Just as Mb appears to be less mobile within the cytosol than previously believed, so also are cytosolic enzymes apparently rather restricted in their intracellular mobility – again this picture is not easily compatible with the concept of the cell as a bag of enzymes whose functions are determined mainly by self-diffusion and substrate diffusion at appropriate rates. With enzyme and Mb translational mobilities reduced to only one tenth of that expected in aqueous solution, diffusion of macromolecules becomes a highly inefficient means of assisting in enzyme–substrate encounter (or in the case of Mb, for assisting O_2 flux through the cytosol).

Third is the argument on metabolite mobility. Because of the complexity of the internal milieu, the translational mobility of even simple molecules may be restricted compared to simple solutions, and this is especially true in the mitochondrial matrix. For example, studies with ^{14}C-labeled Cr (Hochachka and Mossey, 1998) show that CPK is unable to readily equilibrate the entire pool of PCr + Cr in fish white muscle (fast twitch fibers). At the same time, parallel ^{1}H MRS studies (Trump et al., 2001) show that in human muscle *in vivo* the intracellular behaviour of Cr is highly constrained. One set of studies, focussing on the methyl hydrogens, shows that Cr mobility is dependent on metabolic state being three- to fourfold less mobile in ischemic fatigue than in muscle at rest. Another set of studies focussing on the methylene protons found that only in PCr were the methylene protons MRS visible; on PCr conversion to Cr during muscle work, the methylene protons become MRS invisible (in simple solutions MRS cannot distinguish these between PCr and Cr). Taken together these data supply powerful evidence that the behaviour of metabolites *in vivo* may be much more precisely regulated (and certainly much more constrained) than previously expected (for literature in this area, see Hanstock et al., 1999; Trump et al., 2001). Another recent study (Kao et al., 1993) showed that three factors (viscosity, binding and interference from cell solids) could account for translational diffusion of a metabolite-sized analogue in cytosol being decreased to only 27% of the rate observed in water. As in the MRS studies, these workers also demonstrated mobilites that were state-dependent: during osmotic stress (a twofold cell volume increase), when metabolism is known to be increased, there is a correlated (if unexplained) sixfold increase in the apparent translational diffusion coefficient, while rotational diffusion remained constant. The complex and metabolic state-dependent diffusion behaviour of metabolite-sized molecules would not readily facilitate enzyme–substrate encounters as is required for simple solution models of regulated cell function.

Given these constraints, several workers (Wheatley, 2003; Wheatley and Clegg, 1994; Hochachka, 1999b) consider diffusion by itself to be an inadequate, inefficient and minimally regulatable means of delivering carbon substrates and oxygen to appropriate enzyme targets in the cell under the variable conditions and rates that are required *in vivo*. Instead, we favor an hypothesis – almost demanded by the rules imposed by a structured and ordered internal milieu – of an intracellular convection or perfusion system as an elegantly simply resolution of the problem of how substrates (including O_2) and enzymes are brought together. From our present point of view (Hochachka, 1999a), the key advantage of this model is that it easily explains how enzymes and substrates can be brought together and how reaction rates can occur at widely varying rates with minimal change in substrate concentrations. This is the empirical starting point of the paradox in this whole field, which in my opinion has never been satisfactorily explained (for O_2 or for any other intermediate in mainline metabolism). As in the perfusion of organs/tissues such as muscle mentioned above, rates of intracellular metabolic reactions as predicted by this model are simple products of intracellular perfusion rates: the greater the perfusion rates the greater the metabolic rates, with no concomitant changes in substrate concentrations required. In this view, during osmotic activation of metabolic rate, the sixfold increase in metabolite mobility observed (but not explained) could well represent a similarly large increase in intracellular convection. In the case of the MRS data, a fourfold change in Cr mobility in hypometabolic ischemic muscle may well represent a similar change in intracellular convection (this is viewed as a coarse but dominant control, which need not necessarily rule out other fine-tuning control mechanisms, such as those that have so far absorbed much of metabolic research).

For O_2 transport, this view places the function of a half-O_2-saturated, randomly distributed Mb into an entirely different perspective, where the fundamental purpose of an intracellular Mb may be to equalize [O_2] everywhere in the cytosol, thereby ensuring that intracellular convection would always be delivering similar amounts of O_2 per unit volume of cytosol to cytochrome oxidases (and simultaneously minimize or even destroy intracellular O_2 gradients). While this model is consistent with the minimal intracellular O_2 gradients in muscle cells proposed by the Honig and coworkers (Gayeski and Honig, 1986; Honig et al., 1992), it takes on a quite

Table 1. *The path of oxygen*

Model 1 assumes four key processes:

1. Convection: air into the lung.
2. Diffusion: O_2 transfer from aleveoli to pulmonary capillaries.
3. Convection: heart and circulatory transfer of O_2 (+ carbon metabolites and end products) to various tissues and organs.
4. Diffusion: O_2 transfer from capillary blood to tissue intracellular milieu; CO_2 and metabolite exchange.

Model 2 adds:

5. Convection: intracellular circulatory O_2 transfer to mitochondria + numerous substrates to numerous enzymes in cytosolic pathways.
6. Diffusion: final O_2 transfer to cytochrome oxidase sinks (and final enzyme–substrate encounter for cytosolic pathways).

different meaning. Finally, the concept of an intracellular perfusion system supplies purpose and meaning to intracellular movements (motor-driven or otherwise-induced cytoplasmic streaming) that have been mainly ignored by traditional metabolic biochemists to this time. If accepted, the concept of intracellular convection modifies our overall view to include an intracellular component in the chain of convective and diffusive steps in the overall path of O_2 from air to mitochondria (Table 1).

The anatomy of intracellular circulation

According to the best evidence that is currently available, intracellular movement and circulation are largely powered by molecular motors: unconventional myosins running on actin fibers, myosin motor-driven mitochondrial movements on actin tracks, dynein motors running on tubulin tracks, and kinesin motors running on tubulin tracks. Experimental protocols that are used to tease out which systems are operative include monitoring effects of (i) actin fiber disruption (for example, with cytochalasin D), (ii) tubulin disruption (for example, with nocodazole), or (iii) disruption of motors causing cytoplasmic streaming (for example, disruption of such mitochondrial motors stops mitochondrial movement, coincident with a fivefold decrease in cell metabolic rate). Given the above situation, intracellular microfilament distribution may supply us with insight as to possible circulatory pathways.

Interestingly, in slow and fast fibers, intracellular microfilaments are most abundant near the surface and decrease in concentration towards the center. Despite the smaller diameter of slow fibers compared to fast fibers, and their much higher oxidative capacities, microfilament numbers and distribution patterns are similar in slow and fast fibers, implying similar convection capacities. A key insight is that microfilaments extend right through the actomyosin contractile apparatus, both along horizontal and vertical dimensions. Thus, these microfilament distribution patterns in theory supply possible circulatory pathways within the muscle cell, both at the periphery and in the interior of the muscle fiber.

In conclusion, the polarisation illustrated by Model 1 and Model 2 approaches of living cells extends throughout the metabolic regulation field and has caused the field to progress along two distinctly independent pathways, with minimal communication between them. The time may have come when cross-talk between Model 1 and Model 2 approaches may be useful.

During the writing of this paper, I had to undergo chemotherapy for an aggressive lymphoma; we are currently trying to knock it out of the CSF, but success is not assured. Thus this paper should be viewed distinctly as a ‘work in progress’ designed as much to provoke new thinking in the field as it is to provide new answers.

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