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Two research paths for probing the roles of oxygen in metabolic regulation

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Abstract

Tissues such as skeletal and cardiac muscles must sustain very large-scale changes in ATP turnover rate during equally large changes in work. In many skeletal muscles these changes can exceed 100-fold. Examination of a number of cell and whole-organism level systems identifies ATP concentration as a key parameter of the interior milieu that is nearly universally 'homeostatic'; it is common to observe no change in ATP concentration even while change in its turnover rate can increase or decrease by two orders of magnitude or more. A large number of other intermediates of cellular metabolism are also regulated within narrow concentration ranges, but none seemingly as precisely as is [ATP]. In fact, the only other metabolite in aerobic energy metabolism that is seemingly as 'homeostatic' is oxygen - at least in working muscles where myoglobin serves to buffer oxygen concentrations at stable and constant values at work rates up to the aerobic maximum. In contrast to intracellular oxygen concentration, a 1:1 relationship between oxygen delivery and metabolic rate is observed over biologically realistic and large-magnitude changes in work. The central regulatory question is how the oxygen delivery signal is transmitted to the intracellular metabolic machinery. Traditional explanations assume diffusion as the dominant mechanism, while proponents of an ultrastructurally dominated view of the cell assume an intracellular perfusion system to account for the data which have been most perplexing to metabolic biochemistry so far: the striking lack of correlation between changes in pathway reaction rates and changes in concentrations of pathway substrates, including oxygen and pathway intermediates.

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Two classes of models of metabolic regulation

For most of the last 30 years, two general frameworks (for convenience we shall term them I and II) accounting for metabolic regulation in vertebrate muscles have dominated thinking in the field. Framework I considers the cell essentially a bag of enzymes in which simple solution chemistry rules apply; framework II sees the cell as a highly structured system with intracellular ultrastructure incorporating constraints in metabolic processes and in the extreme imposing 3-D order on metabolic function. These two views can be nicely illustrated by considering the vertebrate phosphagen system (1). The most widely accepted framework – framework I –

of phosphagen function in skeletal and cardiac muscles in lower vertebrates and in mammals assumes i) that the total acid-extractable pool of Cr + PCr (termed Cr_{tot}) occurs in aqueous solution and is fully accessible to creatine phosphokinase (CPK), ii) that solution chemistry rules apply globally to 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. Although this is the prevalent view in the current literature and includes other phosphagen systems in invertebrate muscles, it is nevertheless problematic since this model is not easily rationalized with tissue-specific isozyme occurrence, with intracellular localization of specific isozyme forms of CPK, or with intracellular structural constraints. That is why alternative hypotheses (various versions of which we group together for this discussion as framework II) consider i) that the structural organization of phosphagen containing cells physically constrains Cr_{tot}, 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 muscle metabolism. The extent of parallel development of research within these two different frameworks is well illustrated in a comprehensive series of review papers summarizing thinking in this area edited by Saks and Ventura-Clapier (2). This polarization extends throughout the metabolic regulation field and has caused the field to progress along two surprisingly independent paths with minimal communication between them. This paper is an attempt to bring the two approaches into closer harmony.

High precision: the key feature of metabolic pathway integration

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. How such systems are designed and regulated has been a perplexing problem for both physiologists and biochemists. For molecular systems at the cellular level of organization, the individual processes are enzyme-catalyzed reactions; the linked sequences that give meaning to the component reactions are 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 very early on indicated that enzymes in multistep pathways are surprisingly well integrated (see 3). Even in extreme cases, such as in very high capacity metabolic pathways in insect flight muscles or in the electric organ of electric fishes, several hundred-fold flux changes in pathways of ATP demand and ATP 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 (see 3-12 for background literature in this area). These include i) simple feedback and mass action controls, ii) allosteric controls, iii) models involving the regulation of e_o (the concentration of functional catalytic sites by means of alteration in protein interactions (as in actomyosin ATPase), by change in phosphorylation state (as in pyruvate dehydrogenase), by change in redox state (as in V-type ATPases), or by 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. Whichever of these models best accounts for the behaviour of any given metabolic system, the empirical observation is 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. This is observed over and over again, for low capacity and high capacity pathways. A convenient way to illustrate the situation is with a reappraisal of mechanisms of metabolic regulation in human muscle.

Regulation of human muscle metabolism during work

A recent study (13) using noninvasive magnetic resonance spectroscopy (MRS) is rather typical of the kinds of data that form the empirical basis for analysis. In this study of rest, work, and recovery in human calf muscle, intracellular [H^+] was calculated from the exchange-averaged or time-averaged chemical shift difference between monoprotonated and diprotonated phosphate. The concentrations of free ADP, [ADP], were calculated from the equilibrium constant for CPK on the basis of recent studies that assumed it to be 1.77 x 10^9, with free Mg^{2+} taken to be 1 mM and unchanging with exercise; this calculation takes into account the effects of pH on the calculated [ADP]. Also, for these calculations, the study assumed resting [ATP] of 6 mM and Cr_{tot} of 24 mM, 75% phosphorylated (values well within the range expected for muscles of humans and other mammals). Any error in these estimates would change the calculated value of [ADP] but would not alter the fractional changes in concentrations during rest-work-recovery transitions, and it is the latter information which is most relevant to this analysis. ATP turnover rates during different metabolic states could not be determined directly, so the ATP turnover rates were treated as a percentage of the maximum sustainable rate, analogous to percent of maximum voluntary exercise. This is considered reasonable since it is known i) that the ATP turnover rate during sustained submaximal muscle exercise is a direct function of the work rate (14,15), which is why the latter can be used as an index of the former (16), and ii) that during work protocols involving small muscle masses in man (14), the maximum ATP turnover is high (because cardiac output can be preferentially directed to a small working area). Assuming a similar maximum rate of about 100 μmol g^{-1} min^{-1} for these studies indicates muscle exercise intensities equivalent to ATP turnover rates of 20, 30, and 40 μmol g^{-1} min^{-1} in each of the three work episodes; these are about 20, 30, and 40 times muscle RMRs (0.5–1.2 μmol g^{-1} min^{-1} for both slow-twitch and fast-twitch muscles).

PCr and Pi concentration changes in the gastrocnemius were similar to many earlier data (17) for exercising muscles (Figure 1), i.e., declining PCr during exercise with a concomitant rise in Pi, followed by rapid recoveries during each subsequent rest interval. The chemical shift for Pi also showed a modest adjustment, indicating a modest change in the equilibrium between diprotonated and monoprotonated phosphate. The three ATP peaks, in contrast, remained stable throughout the protocol. For the soleus, all [metabolites] seemed more stable during exercise than in the gastrocnemius.

Using ATP turnover rate (assumed to be proportional to muscle exercise intensity) as the independent parameter, the change in ATP demand or in the work rate in the gastrocnemius is linearly reflected in declining PCr concentrations. Since change in Pi is essentially stochiometric with change in PCr, a good relationship is also observed between Pi and ATP turnover rate. However, it will be clear from Figure 1 that the relationship extends far beyond the apparent Km for Pi of mitochondrial metabolism (see Ref. 15); as with the PCr data, a kinetic order of 1 was not observed (Figure 1). For these reasons, it appears that both PCr and Pi reliably reflect the ATP turnover rate demanded by the im-

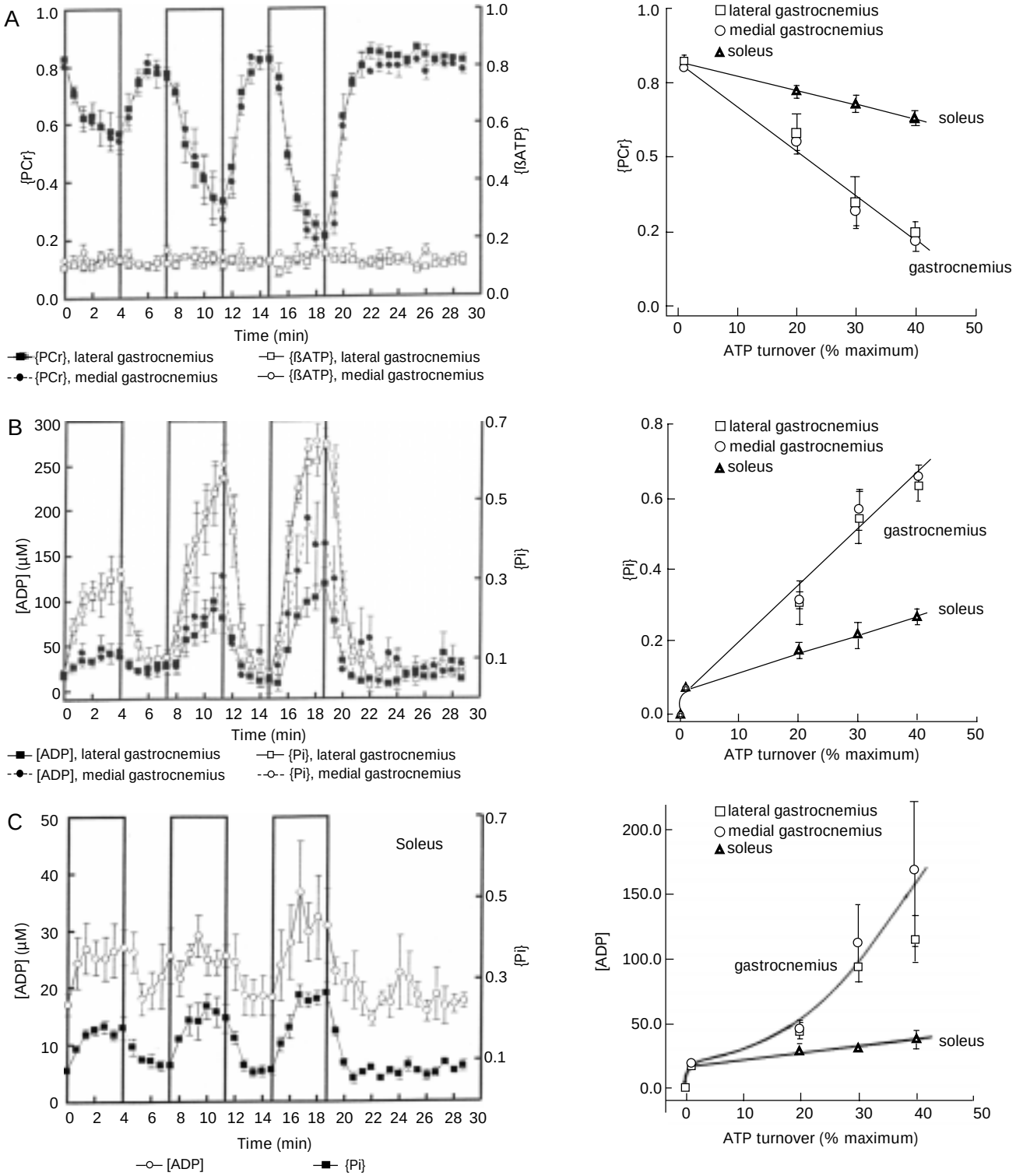

Figure 1 - MRS monitoring of PCr, Pi, and ADP in human gastrocnemius at rest (zero time), at 20, 30, and 40% of maximum sustained work rate (boxed zones), and during recovery in panels A and B, left side; similar data for ADP and Pi are shown for soleus in panel C, left side. Change in muscle ATP turnover rate (as % sustained maximum) is plotted as the independent parameter *vs* {PCr}, {Pi}, and [ADP] in the right panels for gastrocnemius and for soleus. At zero {Pi} and at zero [ADP], the ATP turnover rate is assumed to be necessarily zero. The concentrations of PCr and Pi are given as percent of the sum of [PCr] + [Pi] + [ATP]; the notations, {PCr} and {Pi}, were introduced by Matheson et al. (17) (data modified from Allen et al. (13)).

posed exercises, but do not regulate the ATP turnover rate through effects on mitochondrial metabolism.

In contrast to the simple linear relationships between PCr and Pi concentrations and ATP turnover rates, the relationships with [ADP] and gastrocnemius work intensities are complex. Although the changes in ADP concentration are consistent with some role in metabolic activation, the increase in [ADP] is not adequate to account for work rate changes even if a kinetic order of 2 is assumed (18). This difficulty with ADP as a primary regulator of ATP turnover rate is also noted elsewhere (5,15,19-21). Such rest-work comparisons mean that the fractional changes in ADP concentrations seem to be much lower than the fractional changes in ATP turnover rates in the gastrocnemius muscle. It is unlikely therefore that the former could 'drive' the latter; again, it may be more realistic to view changes in [ADP] as reflecting changes in ATP demand by muscle ATPases (19,22).

For the soleus, the case for regulation of ATP turnover rate by any of these metabolites is even weaker than for the gastrocnemius. The kinetic order is farther from 1 in the case of PCr and Pi - even if both PCr and Pi consistently reflect the differing exercise intensities (Figure 1). With regard to [ADP], the soleus sustains the three exercise intensities at essentially constant ADP concentrations.

In summary, we take these results to mean that, in muscles formed mainly of fast-twitch fibers, ADP and Pi may play a fine-tuning role in regulating ATP turnover rates, but that some other (currently unknown) course-control mechanisms must be operative in controlling large-scale changes in ATP turnover rates during muscle work (21,22). In contrast, in slow-twitch oxidative fibers (which dominate the soleus muscle), neither ADP nor Pi seems to be of any particular regulatory significance - a situation rather reminiscent of the heart of large mammals (5) including humans (23) and in agreement with those few animal studies that have examined this issue in slow-twitch muscle noninvasively (24). In earlier studies (19,21,22), the coupling patterns between ATP-demand and ATP-supply pathways in cardiac and slow-twitch muscles were described as 'tighter' than in fast-twitch muscles because large changes in ATP turnover rates could be sustained with modest (or immeasurable) changes in these key high energy phosphate metabolites.

The general rule is stability of [metabolites] during changes in tissue work

A key point is that the results for human muscle are in no way unusual. Similar conclusions for the adenylates, phosphagen, Pi, and H^+ arise from studies of a wide assortment of animals as well as other human studies. These include invertebrates, fishes and other ectothermic vertebrates, mammals and birds (see Ref. 21). In addition, some of these studies have also analyzed many of the intermediates in specific ATP-supply pathways (such as glycolysis and the Krebs cycle (21); here too changes in [pathway intermediates] are modest (0.5-3-fold) despite large changes in pathway fluxes that are simultaneously sustained by the working tissue.

The implications emerging at this point therefore are i) that [ATP] is almost perfectly homeostatic under most conditions (except under very extreme fatigue conditions) and ii) that other intermediates in pathways of ATP supply or ATP demand are stabilized within less rigorously controlled concentration ranges. Although described as 'relatively homeostatic' in an earlier analysis (25), the % changes in concentrations of intermediates are much lower than the % changes in metabolic rates with which they correlate.

In liver and other tissues, where the difference between RMR and maximally acti-

vated metabolism is modest, a widely accepted model used to explain stable concentrations of adenylates (and other intermediates) at varying ATP turnover rates assumes coordinated control by Ca^{2+} of both ATP-supply and ATP-demand pathways (see Ref. 26 and literature therein). For muscle and heart, these kinds of mechanisms seem inadequate to account for the rate changes observed and the same may apply for the kidney which sustains a very high metabolic scope between ischemic, low flow states and maximally activated, high flow states (5). We have reasoned (21,22) that the simplest model to account for these observations of unanticipated metabolic homeostasis assumes regulation of the concentrations of catalytically active enzymes in pathways of both ATP demand and ATP supply (e_o regulation); this would lead to changes in ATP turnover rates (Figure 1) proportional to the k_{cat} of the enzymes involved with no required change in substrates or products (see Refs. 27,28 for a possible example of this kind of regulation). Another interpretation for enzymes which operate under near-equilibrium conditions assumes that very high catalytic capacities assure sensitive 'high gain' responses to small changes in substrate/product ratios (see 11,12,21,29 for literature in this area). Such near-equilibrium function of CPK is the accepted explanation for the especially precise regulation of [ATP] during rate transitions - the traditional ATP 'buffering' role of CPK (1).

Despite some admitted success of these earlier analyses, for models assuming key regulatory roles for pathway intermediates, the relative homeostasis of most metabolites consistently presents a pretty thorny problem that has not really been acceptably explained: namely, the % change in [putative regulatory intermediate] is always less than the % change in flux required to match the change in ATP turnover rate. Put another way, the kinetic order is usually less than 1, too low to be 'driving' the observed flux or metabolic rate changes. The only metabolite which seems to be an exception is oxygen.

Oxygen delivery is fundamental to metabolic regulation

There is much literature on how oxygen 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 (20,30). For working muscle, suffice to emphasize that numerous studies have found essentially 1:1 relationships between oxygen delivery and muscle work. For example, in recent studies (10,15) using a dog gastrocnemius preparation, we found such a relationship between oxygen delivery and work over an 18-fold change in ATP turnover rate. Later, Hogan et al. (31) used the same preparation to analyze subtle submaximal work changes; these transitions were sustained with immeasurable change in [phosphocreatine] and [ATP]; presumably, therefore, other metabolite concentrations were also stable. Yet through these transitions a 1:1 relationship between change in work and change in oxygen delivery was maintained. As emphasized, these kinds of results are qualitatively similar to those found in many other studies. That is why we and many others in the field accept that oxygen plays a key role in regulating change in ATP turnover (21). But how is the oxygen signal transduced within the cell?

Oxygen signal transduction in working muscle

Unfortunately the answer to this question remains unclear and the only mechanisms proposed by traditional studies in this area assume the Krogh cylinder and calculate smooth diffusion gradients within the cell ending in mitochondrial oxygen sinks. So far this approach has been less than satisfactory for, to unravel the puzzle of how oxygen

delivery translates into effects on metabolism within the cell, we require hard data on intracellular oxygen concentration. The problem is that for most tissues this key parameter remains elusive and unknown. The situation in muscles, however, is more favorable. In this tissue, myoglobin (Mb) supplies a direct intracellular detector of oxygen concentration. Because the reaction Mb + O_2 <- - -> MbO_2 is always in equilibrium (32) with a P50 of about 2-3 torr, $\%MbO_2$ is a direct measure of intracellular [oxygen] whenever O_2 concentrations drop into subsaturation zones (below about 15 μM). Earlier attempts at such measurements with working muscle preparations almost exclusively relied upon near infrared spectroscopy (see 33, for an application of this technique to Weddell seals diving at sea). More recently, MRS is being used to take advantage of a histidine H which is ^1H MRS 'visible' in deoxyMb but being MRS 'invisible' in oxyMb. For the first time, this new technology supplies workers in the field with a noninvasive window on the oxygenation state of Mb-containing muscles in different work and metabolic states. When applied to both working human skeletal muscles (34) and to heart (35) the same striking results arise: essentially stable $\%MbO_2$ (at or near 50% saturation) through large changes in work rate. Along with gold labelling studies showing a random Mb distribution in rat heart and skeletal muscles (Shinn S, McClelland GB and Hochachka PW, unpublished data), the MRS data imply that both $\%MbO_2$ and intracellular [oxygen] remain constant up to the maximum sustainable aerobic metabolic rate of the tissue (34). As CPK serves to 'buffer' ATP concentrations during changes in muscle work, so Mb serves to 'buffer' intracellular oxygen concentrations in different metabolic states. Although most workers in this field would accept that in the midrange of the oxygen saturation curve, Mb should function to buffer intracellular [O_2], the significance of this has not been appreciated. As Carl Honig put it to the author 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 (a scenario denied by the Honig group (36-38)). According to Honig and his coworkers, the structural logic of the capillary-muscle system calls for steep gradients (and localized highest O_2 fluxes) only at the capillary-muscle interface but very shallow gradients within the muscle cell *per se*, as indeed shown by the above later MRS data on $\%MbO_2$ *in vivo*. That is why we (25) accepted the MRS data on $\%MbO_2$ at face value and emphasized in an earlier analysis that, under normoxic conditions, oxygen is perfectly homeostatic in the sense that its concentration is stable even while its flux to cytochrome oxidase can change by orders of magnitude.

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 P50 or K_d range) and intracellular [oxygen] gradients must be quite shallow. The latter point is more fully discussed elsewhere (7,36,38); one of the most important insights emphasized by the Honig, Gayeski and Connett group is 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. Secondly, the low intracellular [oxygen] is powerfully 'buffered' by Mb and remains essentially stable throughout large changes in work and metabolic rates. Nevertheless, $\dot{V}O_2$ and oxygen delivery are closely related, suggesting a key role for oxygen in metabolic regulation.

Given that it is oxygen delivery – not intracellular [oxygen] – which correlates with

work rate, the problem we are left with is how the oxygen signal is transmitted to the machinery of cell metabolism. At this time, we admit that there is no widely accepted answer. When we first recognized this puzzling situation, we proposed a model that postulates an oxygen 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 oxygen (see 21). However, the nature and even existence of such sensing and signal transducing systems remain to be elucidated; and, in any event, they may not be required if the framework II view of O_2 regulation is correct.

Framework II: intracellular structure may require an intracellular perfusion system

Conceptually, the major difference between the above traditional approach to metabolic regulation and framework II is the emphasis placed upon intracellular order and structure. The point of departure for the latter view is that the cell is not a bag of enzymes; instead, it assumes that most metabolic enzymes are largely bound and precisely localized within the cell and it assumes that important functional consequences arise. Time and space will not allow a detailed review of the evidence for this position. Suffice to emphasize that it arises from several approaches and the overall hypothesis is constructed from three different lines of evidence favoring perfusion and of argument not favoring diffusion. First and most fundamental is the structural argument: ultrastructural, histochemical, and cytochemical studies do not indicate the cell as a static bag of enzymes, but rather a 3-D 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 (39)), 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 1-60 μm/s (39)). In contrast to what might be expected of a bag of enzymes, 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 (see 40 for a recent study of aldolases) show intracellular binding to specific intracellular sites (This already has been alluded to for CPKs in introducing the problem (also see 41)). 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.

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 1/10th that found in simple solutions (42), to values of about 1/2 (43). Interestingly, the latter MRS study estimated rotational diffusion, while Juergens et al. (42) estimated translational diffusion; as indicated below, these may change independently (44). Be that as it may, cytosolic enzymes also apparently are highly restricted in their intracellular mobility (40,42) – again this picture is not easily compatible with the concept of the cell as a bag of enzymes. Order and structure seem to be constraining the intracellular behavior of macromolecules and their restricted mobilities would not facilitate the kind of enzyme-substrate encounters predicted by simple solution models of cell function. In fact, recent studies indicate severe disruption of function if the 3-D organ-

ization of the so-called cytosolic enzymes is disrupted (see note added in publication process).

Third is the argument on metabolite mobility: because of the complexity of the internal milieu, the translational mobility even of simple molecules may be restricted compared to simple solutions (44), and this is especially true in the mitochondrial matrix (45). A recent study dissected different contributions to limiting mobility of intracellular metabolites. Compared to water, hindrance to translational diffusion in the cytoplasm could be attributed to three independent factors – viscosity, binding, and interference from cell solids. Thus fluid-phase cytoplasmic viscosity in the fibroblasts used in the study was nearly 30% greater than water. Nonspecific, transient binding of small solutes by intracellular components of low mobility decreased metabolite mobility by about 20%. And, translational diffusion of small solutes was hindered 2.5-fold by collisions with cell solids compromising about 15% of the isosmotic cell volume. Together, these three factors could account for translational diffusion in cytosol that was decreased to only 27% of that observed in water (44). Interestingly, these studies also demonstrated that during osmotic stress (cell volume increasing to 2 times isosmotic volume), the relative translational diffusion coefficient increased by about 6-fold while the rotational diffusion constant remained unchanged. In short, in these studies as well, order and structure seem to dominate the intracellular behavior of micromolecules such as substrates (including oxygen) of energy metabolism, and the constraints on diffusion would again not readily facilitate enzyme-substrate encounters as required for simple solution models of cell function.

Given that enzymes are structurally localized and not free to readily diffuse about, and that substrates are also relatively restricted compared to simple solutions, workers in this area (39) consider diffusion by itself to be an inadequate, inefficient, and minimally regulatable means of delivering carbon and oxygen to appropriate enzyme targets in the cell under the variable conditions and rates that are required *in vivo*. Instead, an intracellular convection system is proposed as an elegantly simple resolution of the question of how substrates (including O_2) and enzymes are brought together. From the point of view of this essay, 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. As in the perfusion of organs mentioned above, in this model the rate of intracellular metabolism is a simple product of intracellular perfusion rate: the greater the perfusion rate the greater the metabolic rate with no concomitant change in substrate concentrations required – a principal and a coarse control mechanism well appreciated by physiologists at least since the time of Fick (of course, this need not rule out the other control mechanisms, the kinds that have so far absorbed much of metabolic research).

For O_2 transport, this view places Mb function into an entirely different perspective where the fundamental purpose of an intracellular Mb may be to equalize [O_2] everywhere in the cytosol – this would assure that intracellular convection would always be delivering similar amounts of O_2 per unit volume of cytosol to cytochrome oxidases. From this point of view, the 'buffering' function of Mb, the function of a half O_2 saturated, randomly distributed Mb, is to assure a similar [O_2] everywhere in the cytosol (and simultaneously to minimize or even destroy intracellular O_2 gradients). While this model is consistent with the minimal intracellular O_2 concentration gradients in muscle cells proposed by the Honig, Connett, and Gayeski group, it takes on a quite a different meaning. Finally, the concept of an intracellular perfusion system supplies pur-

pose and meaning to intracellular movements (motor-driven or otherwise induced cytoplasmic streaming) which to this point in time have been pretty well ignored by traditional metabolic biochemists.

Diffusion, of course, is a limited solution to limited problems. Earlier (21) we pointed out that, in the up-regulation of metabolic capacities of skeletal and heart muscles (for example in organisms such as the hummingbird (46-48)), the higher the O_2 fluxes required, the less and less dependent upon diffusion muscle metabolic organization seems to become. And, to emphasize the limitations of diffusion (the need for convection systems), even earlier Schmidt-Nielsen (49) pointed out that if diffusion were the only means of O_2 transport in humans, an O_2 molecule travelling at 10 μm/ms would take 3 years to get from the lungs to the foot. For these kinds of reasons, physiologists have generally agreed that organisms get around diffusion limitation problems of O_2 transport by relying on convection systems: ventilation in the lungs and circulation to the tissues, interspersed with diffusion-based steps along the way. If accepted, the concept of intracellular convection modifies our overall view to include an intracellular component to the chain of convective and diffusive steps in the overall path of O_2 from air to mitochondria.

When considering the concept of intracellular convection, early pioneers in this field may be prone to over-enthusiastic pressing of their case; this is understandable, since it seems to explain so much previously puzzling data so easily (39,50). Nevertheless, there clearly remain critical functions that are largely or solely diffusion based, so the understandable over-enthusiasm with which framework II proponents minimize the importance of diffusion in energy metabolism puts them at risk of throwing the baby out with the bathwater. What seems to be required for the future to finally assemble a model which can realistically explain a realistic working range of metabolic systems is an opening up of channels of communication between the above two very different views of metabolic regulation.

Note added in publication process. A good recent example of the role of intracellular localization of enzymes 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. (51) 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 3-D organization is part and parcel of *in vivo* regulated operation of the pathway.

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