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# Review

# Myoglobin function reassessed

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**Summary**

**The heart and those striated muscles that contract for long periods, having available almost limitless oxygen, operate in sustained steady states of low sarcoplasmic oxygen pressure that resist change in response to changing muscle work or oxygen supply. Most of the oxygen pressure drop from the erythrocyte to the mitochondrion occurs across the capillary wall. Within the sarcoplasm, myoglobin, a mobile carrier of oxygen, is developed in response to mitochondrial demand and augments the flow of oxygen to the mitochondria. Myoglobin-facilitated oxygen diffusion, perhaps by virtue of reduction of dimensionality of diffusion from three dimensions towards two dimensions in the narrow spaces available between mitochondria, is rapid relative to other parameters of cell respiration. Consequently, intracellular gradients of oxygen pressure are shallow, and sarcoplasmic oxygen pressure is nearly the same everywhere. Sarcoplasmic oxygen pressure, buffered near 0.33 kPa (2.5 torr; equivalent to approximately 4 μmol $l^{-1}$ oxygen) by equilibrium with myoglobin, falls close to the operational $K_m$ of cytochrome oxidase for oxygen, and any small increment in sarcoplasmic oxygen pressure will be countered by increased oxygen utilization. The concentration of nitric oxide within the myocyte results from a balance of endogenous synthesis and removal by oxymyoglobin-catalyzed dioxygenation to the innocuous nitrate. Oxymyoglobin, by controlling sarcoplasmic nitric oxide concentration, helps assure the steady state in which inflow of oxygen into the myocyte equals the rate of oxygen consumption.**

Key words: myoglobin, oxygen, facilitated diffusion, dimensionality in diffusion, heart, red skeletal muscle, nitric oxide, mitochondria, cytochrome oxidase, Krogh cylinder.

## Introduction

Myoglobin, a mobile carrier of oxygen, is developed in red muscle in response to mitochondrial demand for oxygen (Millikan, 1939; Wittenberg, 1970; Williams and Neufer, 1996) and transports oxygen from the sarcolemma to the mitochondria of vertebrate heart and red muscle cells (Wittenberg and Wittenberg, 1989; Takahashi and Doi, 1998). Likewise, leghemoglobin, a protein similar to myoglobin but with 10-fold greater oxygen affinity, transports oxygen from the cell membrane of the central cells of the legume root nodule to the symbiosomes, which are membrane-bound intracellular organelles housing the bacteroids, the intracellular nitrogen-fixing form of the bacterium *Rhizobium* (Appleby, 1984). A similar system is found in neural cells, including the photoreceptors, of the vertebrate retina. Here, neuroglobin, a cytoplasmic hemoglobin that has the classic globin fold but is otherwise only distantly related to myoglobin (Pesce et al., 2002), apparently mediates oxygen supply to the mitochondria (Schmidt et al., 2002).

Within the sarcoplasm of the cardiac myocyte or of red skeletal muscle fibers, translational diffusion of oxymyoglobin molecules, each carrying pickaback a diatomic oxygen molecule (with an equal back flow of deoxymyoglobin molecules), is believed to support a flux of oxygen from the sarcolemma to the mitochondrial surface. The molecular mechanism of this process, which has been called myoglobin-facilitated oxygen diffusion, has been elucidated for solutions of hemeproteins that bind oxygen reversibly (Wyman, 1966; Wittenberg, 1966, 1970; Murray, 1971, 1977; Keener and Sneyd, 1998). The mechanisms of intracellular oxygen diffusion have not been described. We note that oxygen is very insoluble in water, and that the ratio of myoglobin-bound oxygen to free oxygen approximates 30:1 within working vertebrate heart or muscle cells at 37°C. Accordingly, a large fraction of the oxygen flux through the cytoplasm must be myoglobin supported. More impressively, in the legume root nodule the cytoplasmic leghemoglobin concentration may exceed millimolar; the dissolved oxygen concentration is vanishingly small ($10^{-8}$ mol $l^{-1}$) and the ratio of leghemoglobin-bound oxygen to free oxygen exceeds $10^5$:1. Essentially all of the oxygen flux must be leghemoglobin mediated.

Here, we describe myoglobin-augmented oxygen supply to heart and red muscle, taking into account their three-dimensional structures and the elevated concentration of myoglobin in the cytoplasmic domain to which it is restricted and recognizing the large area of mitochondrial surface available for oxygen diffusion. [A mathematical formulation of oxygen diffusion in the cardiac myocyte will be presented elsewhere.] Heart and muscle, having available an almost unlimited supply of oxygen, actually operate at controlled low oxygen pressure, at or near 0.33 kPa (2.5 torr), where myoglobin is about half-saturated with oxygen. Partial saturation of myoglobin enables oxymyoglobin to play a pivotal role; by converting endogenous nitric oxide to the innocuous nitrate, oxymyoglobin controls the level of nitric oxide (NO) within the cell. This, in turn, may control both the rate of capillary oxygen delivery to the cell and the rate of oxygen utilization by cytochrome oxidase.

## Formulations of oxygen diffusion in muscle

Present descriptions of oxygen diffusion/transport in tissues originate from the studies of Krogh, Hill and Jeffries Wyman, to whom this essay is dedicated.

Krogh (1919a,b) and later Hill (1928) considered that oxygen flowed from the capillary down a continuous gradient of oxygen pressure towards a plane (or cylinder), about half-way between two capillaries, where oxygen pressure would be minimal. Groebe (1995) has expanded this model, and his treatment is used in the recent calculations of Gros and collaborators (e.g. Jurgens et al., 2000). However, the Krogh cylinder model is not in accord with present day concepts of oxygen gradients in muscle. Currently, gradients of oxygen pressure around the capillary are thought to be discontinuous. A large oxygen pressure drop across the capillary wall is followed by a very shallow gradient across the sarcoplasm. Furthermore, the simple model of diffusion between two concentric cylinders is not in accord with electron micrographs showing a convoluted oxymyoglobin diffusion path and a very large area of mitochondrial surface.

Wyman (1966), working with Wittenberg's (1966) data, formulated a description of the, then relatively new, phenomenon carrier-mediated oxygen transport. Wyman's equation was solved analytically by Murray (1971, 1977; see Keener and Sneyd, 1998) and used to construct profiles of oxygen concentration within muscle cells. Independently, Kreuzer and Hoofd (1970) devised a nearly identical equation and solved it with computer assistance (reviewed in Kreuzer, 1970). It would be of great interest to adapt Wyman's description to our current understanding of muscle and cytoplasmic structure.

## The requirement for myoglobin

Any discussion of myoglobin function must begin with Millikan's (1939) notable review in which he assembles an impressive body of knowledge to establish that myoglobin is formed adaptively in tissues in response to the demand for oxygen and that myoglobin contributes to the oxygen supply of these tissues. Subsequent work (reviewed in Wittenberg, 1970; Wittenberg and Wittenberg, 1989) bolsters Millikan's conclusion that: '*muscle hemoglobin is generally found in large quantities in those muscles requiring slow, repetitive activity of considerable force…and whose action must be maintained over long periods*'. Possibly, low tissue oxygen pressure may initiate myoglobin formation. Certainly, myoglobin messenger RNA is elevated under conditions eliciting myoglobin formation (Williams and Neufer, 1996). Myoglobin is necessary to support cardiac function in the fetus (Meeson et al., 2001).

Blockade of myoglobin function in mammalian or avian skeletal muscle sharply decreases oxygen uptake and work output (reviewed in Wittenberg and Wittenberg, 1989). Blockade of myoglobin mimics hypoxia, monitored by the ratio of mitochondrial NADH/NAD in isolated cardiac myocytes stimulated electrically to contract (White and Wittenberg, 1993). Blockade of leghemoglobin function sharply decreases bacteroidal oxidative phosphorylation within the soybean (*Glycine max*) root nodule (Bergersen et al., 1973). Shallow radial gradients of oxygen pressure, visualized as myoglobin oxygenation or NAD(P)H reduction in the central region of isolated cardiac myocytes (Takahashi et al., 1998, 2000; Takahashi and Asano, 2002), are abolished by blockade of myoglobin function.

### *Mice without myoglobin*

Knockout of the myoglobin-encoding gene (Garry et al., 1998; Godecke et al., 1999) induces multiple compensatory mechanisms that tend to steepen the oxygen pressure gradient to the mitochondria (Godecke et al., 1999; Grange et al., 2001; Meeson et al., 2001). These include a higher capillary density, smaller cell width, elevated hematocrit and increased coronary flow and coronary flow reserve. Transitions of type I to type II fiber types and increased expression of hypoxia-inducible transcription factors (HIF)-1α and HIF-2 (endothelial PAS domain protein), heat shock protein 27 and endothelial growth factor were also observed, and these tend to increase energy supply when oxygen is limiting. Taken together, these compensations demonstrate that myoglobin, when present, assures the oxygen supply of normal heart and muscle.

Experiments in which myoglobin function is abolished acutely suffer the criticism that the inhibitor or blocking agent may have effects other than those intended. Hearts from myoglobin-knockout mice served as the ideal control in a study of acute carbon monoxide inhibition of myoglobin in the isolated mouse heart (Merx et al., 2001). The results provide conclusive direct evidence that myoglobin is required to assure oxygen flow from the vasculature to mitochondrial cytochrome oxidase.

### *Fish without myoglobin*

Antarctic ice-fishes of the family Channichthyidae lack blood hemoglobin and circulating red blood cells. Some

species lack cardiac myoglobin as well. The mechanical performance of isolated perfused hearts from two very similar, congeneric channichthyids show little difference at normal work loads, but that of the species with myoglobin is far more able to maintain cardiac output in the face of the additional insult of increased aortic arterial pressure (Acierno et al., 1997; Sidell, 1998). Channichthyid fish without myoglobin served as a control of the effects of nitrite in an experiment demonstrating decreased cardiac function following blockade of cardiac myoglobin (Acierno et al., 1997).

### Myoglobin supports oxidative phosphorylation

Essentially all of the oxygen consumed by skeletal muscle and heart is taken up by cytochrome oxidase. Myoglobin is developed in skeletal muscle more or less in proportion to the cytochrome oxidase content of the muscle (Fig. 1; Lawrie, 1953).

Most of the oxygen used by the soybean root nodule is consumed by the terminal oxidases of intracellular bacteroids, the plant mitochondria being relatively sparse. In turn, most of the ATP produced by bacteroidal terminal oxidases is consumed by the intrabacterial enzyme nitrogenase; hence, nitrogenase activity can be used as a measure of the rate of ATP formation. Carbon monoxide blockade of leghemoglobin in the living, plant-attached nodule causes nitrogenase activity to collapse (Bergersen et al., 1973). In an *in vitro* system using isolated bacteroids, the rate of ATP production, measured as nitrogenase activity, was proportional to the concentration of leghemoglobin added (Wittenberg et al., 1974). These experiments show that ATP generation is leghemoglobin dependent.

### Myoglobin

Myoglobin is a relatively small protein ($M_r$ 17 600) that is little affected by environmental conditions [with the exception of temperature and high concentrations of lactate (Giardina et al., 1996)]. The molecules are slippery in the sense that they slide past one another with little frictional interaction (Riveros-Moreno and Wittenberg, 1972; Veldkamp and Votano, 1976).

Myoglobin in the heart is generally close to 200–300 μmol $kg^{-1}$ wet mass tissue but may reach 400–500 μmol $kg^{-1}$ wet mass in skeletal muscles. Since myoglobin is excluded from mitochondria (35% of cell volume) and the sarcoplasmic reticulum (4% of the cell volume), the concentration in the remaining volume of the heart cell becomes 330 μmol $l^{-1}$. The extent to which myoglobin penetrates the myofibrillar volume (47% of the cell volume) is not known, but, in view of the intimate association of mitochondria with the contractile elements of cardiac muscle (Fig. 2A), we shall assume that it must. Leghemoglobin, 380 μmol $kg^{-1}$ wet mass in nodules, is at a concentration of approximately 700 μmol $l^{-1}$ in the space to which it is confined (Wittenberg et al., 1996). Myoglobin concentration increases with the work to which the muscle is put and should be regarded as optimized for the particular muscle at a particular rate of work output.

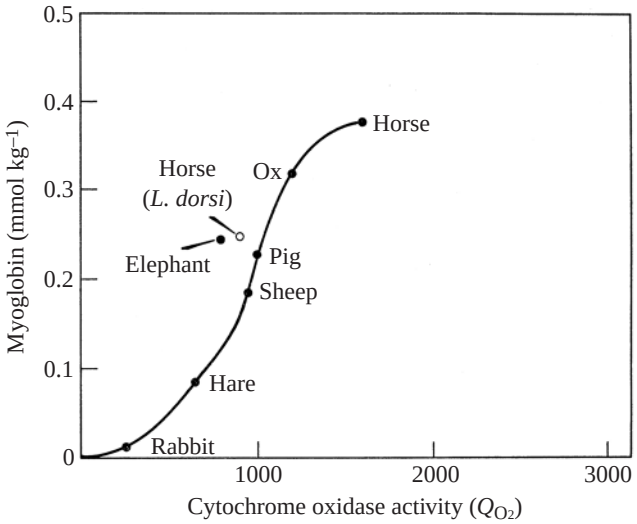


Fig. 1. Relation of myoglobin concentration in muscles of various animals to their cytochrome oxidase activity. Modified from Lawrie (1953).

The rates of reaction of myoglobin/leghemoglobin with oxygen are subject to natural selection. For instance, Gibson et al. (1989) have found that an array of disparate leghemoglobins have strikingly similar, rather slow rates of oxygen dissociation. These rates determine the length of the path explored during the random walk of an oxymyoglobin molecule, as discussed below. The rate constants for oxygen combination are close to the maximum achievable. Oxygen affinities in the nanomolar range are achieved, and the oxygen pressure in the functioning nodules, close to 0.03 kPa (0.02 torr; equivalent to approximately 10 nmol $l^{-1}$) provides a suitable environment for the highly oxygen-intolerant bacterial nitrogenase system.

Oxygen affinity is also subject to genetic selection pressure. The oxygen affinities and oxygen dissociation rate constants of myoglobins from predacious, oceanic fish that maintain muscle temperatures well above ambient are similar to those of a related fish whose muscle operates at cool ambient temperature, when compared at the operating temperature of the muscle (Cashon et al., 1997; Marcinek et al., 2001).

### Diffusivity of myoglobin

Until recently, the self-diffusion coefficient of myoglobin, in solutions whose concentration was comparable to that of proteins in the cell, was used in calculations of oxymyoglobin diffusion in tissue. These values have now been supplanted by the significantly smaller values measured by microinjection and photobleaching experiments in living muscle (Baylor and Pape, 1988; Jurgens et al., 1994, 2000; Papadopoulos et al., 1995, 2000, 2001). The value found in photobleaching experiments ($1.2\times10^{-7}$ $cm^2$ $s^{-1}$ at 22°C or $2.1\times10^{-7}$ $cm^2$ $s^{-1}$ at 37°C) is about one-tenth that in dilute solution

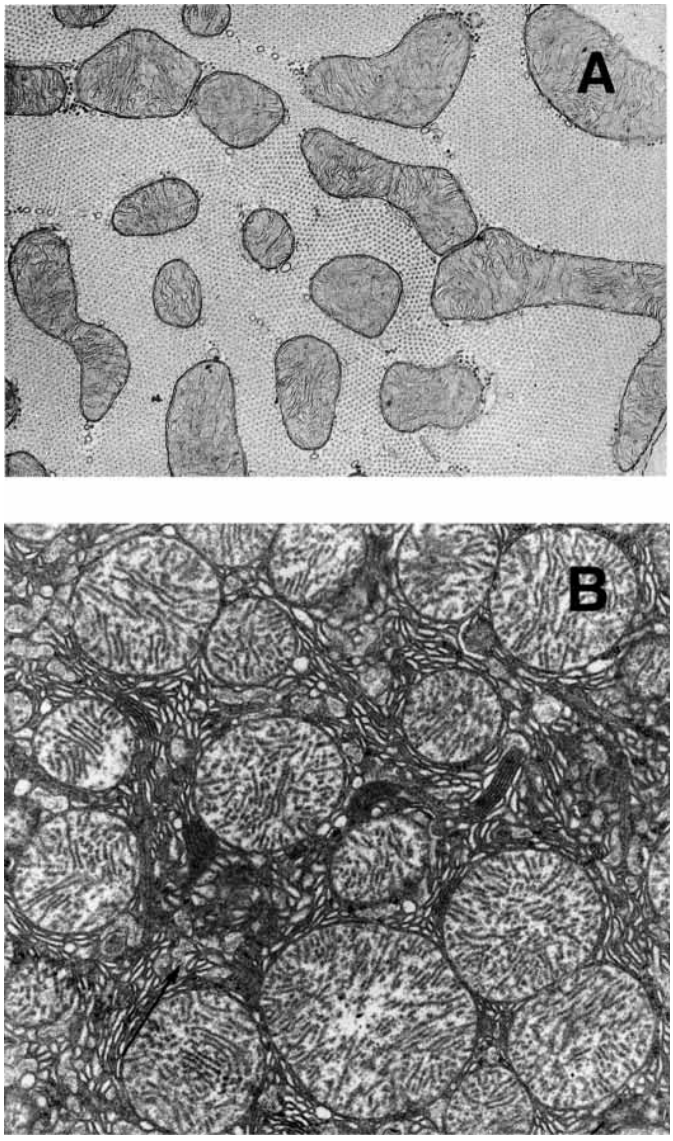


Fig. 2. (A) portion of a myocyte from the ventricular papillary muscle of the cat. Reproduced with permission from Fawcett (1966). (B) A portion of a modified striated muscle cell from the heater organ of the eye of the blue marlin. Reproduced with permission from Block and Franzini-Armstrong (1988).

($11.3\times10^{-7}$ $cm^2$ $s^{-1}$ at 20°C) and about one-fifth that often used in earlier calculations of the magnitude of facilitated diffusion. The identical values found for the diffusion coefficients of myoglobin in the longitudinal and radial directions in the muscle fiber (Papadopoulos et al., 2001), together with nearly unimpeded rotational diffusion (Wang et al., 1997), suggest that myoglobin may diffuse within an aqueous phase that is continuous for long distances in the cytoplasm. Furthermore, the measured value is not affected by contraction of the muscle between measurements and is the same in heart and red skeletal muscle. We cannot distinguish whether the impediment to myoglobin diffusion arises from friction between molecules or whether it is due to obstruction of the diffusing path by molecules or structures in the cytoplasm, with consequent tortuosity of the diffusion path.

## The diffusion path

The diffusion path for oxymyoglobin is always to some degree tortuous, and we must abandon the idea of a simple, unimpeded linear path implied in the Krogh cylinder model. For instance, mitochondria, from which myoglobin is excluded, comprise approximately 35% of the cell volume of mammalian heart, and any transept of a heart cell will be interrupted many times by mitochondria (Fig. 2A). Mitochondrial volume is optimized and, for instance, increases in cold-acclimated fish (Egginton and Sidell, 1989). Leghemoglobin is excluded from the symbiosomes (Wittenberg et al., 1996); the leghemoglobin-accessible volume is less than half the volume of the root nodule cell, and the diffusion path is indeed tortuous (Studer et al., 1992). An extreme is reached in eye muscles of blue marlin, which are modified for heat production (Block and Franzini-Armstrong, 1988; Fig. 2B). Here, mitochondria comprise approximately 70% of the cell volume; sarcoplasmic reticulum and T-tubules appear to occupy most of the balance, and myoglobin must be constrained to the tiny remaining space, where it may reach a concentration of 4 mmol $l^{-1}$ in the cytoplasm proper, a truly impressive value (B. A. Block and J. B. Wittenberg, unpublished data). Any model for myoglobin-augmented oxygen transport in muscle must be compatible with this structure.

Because of its simpler cytoarchitecture, heart muscle is favored over skeletal muscle for construction of a model of oxygen inflow. [A mathematical formulation of oxygen diffusion in the cardiac myocyte will be presented elsewhere.] Within the heart muscle cell, mitochondria are arrayed in long columns parallel to the long axis of the cell and often about one sarcomere in length. In cross-sections of heart muscle, the columns of '*…mitochondria are not randomly distributed but are so evenly spaced as to suggest that each serves only a very limited area of the myofilament mass immediately surrounding it.*' (Fawcett and McNutt, 1969; Fig. 2A). Simple diffusion may suffice to distribute ATP, newly generated in the mitochondria, throughout this limited area (Meyer et al., 1984). Morphometric analysis, assuming a hexagonal array of capillaries, detects increased density of the mitochondrial columns in a band about 4–5 μm removed from each capillary (Kayar et al., 1986).

The oxygen dissociation rate constant, together with the diffusion coefficient, determine the distance traveled in the random walk of myoglobin molecules during the time that an oxygen molecule is resident. A remarkable feature of the random walk is that '*…a diffusing particle that finds itself in a given region of space is destined…to wander around that region for a time, probing it rather thoroughly before wandering away for good.*' (Berg, 1983). The mean radii of the region of cytoplasm explored by oxymyoglobin molecules during the time that an oxygen molecule is resident are given

Table 1. *Estimated mean displacement of oxymyoglobin molecules in the sarcoplasm during the time that an oxygen molecule is resident*

| Protein | Temperature (°C) | Oxygen dissociation rate ($s^{-1}$) | $t_{1/2}$ (ms) | Mean displacement[a] (μm) |
|---|---|---|---|---|
| Sperm whale myoglobin | 20 | 12 | 60 | 20 |
| | 37 | 60 | 12 | 12 |
| Blue fin tuna myoglobin | 25 | 104 | 6.7 | 8 |
| Blue marlin myoglobin | 25 | 84 | 8.3 | 9 |
| Soybean leghemoglobin | 20 | 5.6 | 120 | 30 |

[a]Calculated from the relation $<r^2>=6Dt$, where $r$ is the displacement in time $t$, and time $t$ is taken as the half-time for oxygen dissociation. The diffusion coefficients (D) used are those given by Papadopoulos et al. (2000).

in Table 1. These radii are large compared with the narrow spaces available between mitochondria in muscle or symbiosomes in the root nodule. If diffusing oxymyoglobin molecules are for the most part reflected off the surfaces of the confining mitochondria, the volume explored will be flattened from something like a sphere to something more like a disc, whose radius will be greater than that of an unconstrained sphere. Oxymyoglobin molecules will be displaced further in the plane of the confining mitochondrial surface during the residence time of an oxygen molecule, and myoglobin-facilitated oxygen diffusion will be accelerated. An elegant description of this effect (Adam and Delbruck, 1968) is that the dimensionality of oxymyoglobin diffusion is reduced from three dimensions towards two dimensions. Two-dimensional diffusion is much more rapid than three-dimensional diffusion.

## Myoglobin operates in states of partial oxygenation

The striking fact is that muscles and plant root nodules, having available an almost limitless supply of oxygen, actually operate at those low oxygen pressures where myoglobin or leghemoglobin are partially desaturated with oxygen. These states of partial oxygenation resist change in the face of changing workload or oxygen availability.

Leghemoglobin is approximately 80% deoxygenated in the living root nodule (Appleby, 1969), and the fractional oxygenation of leghemoglobin in the living, plant-attached nodule responds immediately to a step change in ambient oxygen pressure but reverts in minutes to the original value. (Klucas et al., 1985).

Millikan (1937), using an oximeter that he had devised for the purpose, reported extensive deoxygenation of myoglobin in skeletal muscle *in situ* as it was brought into maximal contraction. Subsequent studies of the beating heart, either *in situ* or saline perfused (e.g. Fabel and Lubbers, 1965; Hassinen et al., 1981), fully confirm Millikan's finding. Cryomicrospectrophotometry of rapidly frozen tissue permits quantification of myoglobin saturation (Voter and Gayeski, 1995). Myoglobin in the *in situ* beating heart is maintained near half-saturation in the face of a 20-fold change in work output, a 5-fold change in heart rate and a 2-fold change in arterial oxygen content (Gayeski and Honig, 1991). Likewise, myoglobin saturation in red skeletal muscle, working near maximum sustainable oxygen consumption, is controlled near 50% saturation (Gayeski and Honig, 1986, 1988).

A quantitative nuclear magnetic resonance (NMR) study showed that myoglobin saturation (near 76%) in the blood-perfused isolated heart was held invariant in the face of an 8-fold increase in heart rate (Jelicks and Wittenberg, 1995). A non-invasive NMR study of hard-working human leg muscle reports 50% deoxygenation of myoglobin, with sarcoplasmic oxygen partial pressure ($P_{O_2}$) near 0.32 kPa (2.4 torr; recalculated from Richardson et al., 2001, taking $P_{50}$=0.32 kPa at 37°C; Schenkman et al., 1997). On the other hand, the consensus of recent NMR studies of the *in situ* beating heart is that cardiac myoglobin may be only about 10% deoxygenated under basal conditions (Zhang et al., 2001).

## The sarcolemmal boundary

The capillary endothelial surface offers by far the smallest cross-sectional area in the diffusion path for oxygen from blood to cytochrome oxidase, and a large oxygen pressure difference is expected at this point. Experiments by Cole et al. (1982), showing that partially desaturated myoglobin hastens the entry of oxygen from a gas phase into a myoglobin-containing watery solution, serve as a model for the sarcolemmal boundary of the sarcoplasm. Entering oxygen combines with deoxymyoglobin adjacent to the sarcolemma, and the oxymyoglobin thus generated diffuses into the bulk of the sarcoplasm, dissipating the local concentration of oxygen that would otherwise result near the sarcolemma.

Very shallow gradients of oxygen pressure encountered within the sarcoplasm of isolated cardiac myocytes (Katz et al., 1984; Wittenberg and Wittenberg, 1985) suggest that the largest part of the oxygen pressure drop from the erythrocyte to the mitochondria of cardiac and red skeletal muscle, approaching 2.7 kPa (20 torr), must be ascribed to the pressure drop across the capillary wall (Landis and Pappenheimer, 1963; Wittenberg and Wittenberg, 1989). Partially desaturated sarcoplasmic myoglobin, observed only micrometers away from capillaries, once again indicates that the pressure drop from erythrocyte to sarcoplasm is large (Gayeski and Honig, 1986, 1988; Honig et al., 1984, 1992).

The large oxygen pressure difference across the capillary wall, say 2.7–3.3 kPa (20–25 torr), will not be much affected by small changes in sarcoplasmic oxygen pressure. Accordingly, control over rate of oxygen entry into the myocytes will be vested almost entirely in the number of capillaries open at any one time (Krogh, 1919a,b).

### The mitochondrial boundary

The surface area of the mitochondria, which is 30- to 150-fold greater than the area of capillary lumen serving each cell (Clark et al., 1987; Wittenberg and Wittenberg, 1981, 1989), is by far the largest area in the diffusion path for oxygen. Accordingly, the oxygen pressure difference across the mitochondrial surface need only be small, and the oxygen pressure experienced by cytochrome oxidase will closely approach sarcoplasmic oxygen pressure. Myoglobin does not interact detectably with the mitochondrial surface. The only requirement to sustain respiration of isolated cardiac mitochondria is maintenance of a sufficient, myoglobin-maintained oxygen pressure at the mitochondrial surface (J.B.W. and B.A.W., unpublished).

### Tissue oxygen supply is not limiting

Models of oxygen flow in muscle often assume that oxygen supply to mitochondria is barely sufficient and that diffusion of oxygen limits muscle work output. This is not the case in the normally functioning heart nor in skeletal muscles operating below maximal work output (Jobsis and Stainsby, 1968; Gayeski et al., 1987; Murakami et al., 1999; Richmond et al., 1999; Zhang et al., 1999). Furthermore, oxidative phosphorylation in cardiac muscle is independent of oxygen pressure above a low 'critical' $P_{O_2}$ (Jobsis and Stainsby, 1968; Wittenberg, 1970), and phosphocreatine levels in the *in situ* dog heart decrease only when sarcoplasmic oxygen pressure falls below the normal 0.67 kPa (5 torr; Zhang et al., 2001).

### Sarcoplasmic oxygen pressure gradients

Gradients of oxygen pressure within the sarcoplasm of isolated cardiac myocytes are found to be very shallow, and sarcoplasmic oxygen pressure, within the limits of measurement, is the same everywhere. For instance, the volume-average sarcoplasmic oxygen pressure of resting isolated cardiac myocytes, probed spectroscopically as myoglobin oxygenation, differs only slightly from extracellular $P_{O_2}$ (Wittenberg and Wittenberg, 1985) and, in turn, does not differ from outer mitochondrial membrane oxygen pressure, as measured by the enzyme monoamine oxidase (Katz et al., 1984).

Furthermore, the sarcoplasmic oxygen pressure that supports half-maximal mitochondrial function in isolated cardiac myocytes (Wittenberg and Wittenberg, 1985) does not differ from that required to support half maximal respiration of isolated cardiac mitochondria (J.B.W. and B.A.W., unpublished). [A different relationship between oxygen uptake and ambient $P_{O_2}$ is reported for isolated cardiac myocytes in transient states of changing $P_{O_2}$ (Rumsey et al., 1990).] Independently, Gayeski et al. (1987) conclude that the $P_{O_2}$ experienced by cytochrome oxidase is virtually identical to mean sarcoplasmic oxygen pressure of red muscles working near maximal oxygen uptake. Finally, the demonstration by Vanderkooi et al. (1990), using two luminescent probes, one membrane bound and one in solution, that the oxygen pressure in the mitochondrial membrane proper does not differ from that in the immediately adjacent solution, completes the proof that the oxygen pressure experienced by mitochondrial cytochrome oxidase is very nearly the same as that established in the equilibrium between sarcoplasmic myoglobin and oxygen.

### Cytochrome oxidase

Since myoglobin cannot cross the mitochondrial outer membrane, cytochrome oxidase, located in the inner mitochondrial membrane and cristae, must be supplied by dissolved oxygen diffusing from the sarcoplasm. The exceedingly thin mitochondrial outer membrane will scarcely impede oxygen diffusion (Vanderkooi et al., 1990).

Cytochrome oxidase is only partially (approximately 10%) reduced in resting cardiac myocytes (Wittenberg and Wittenberg, 1985). However, cytochrome oxidase (as $Cu_A$) in red skeletal muscle contracting *in situ* becomes reduced in proportion to increasing workload to about 90% reduction at maximum oxygen uptake (Duhaylongsod et al., 1993; Boushel and Piantadosi, 2000). If this effect reflects recruitment of motor units, we may consider that the oxidase is largely reduced in each contracting myocyte. The effect is to accelerate the combination of oxygen with cytochrome oxidase as respiratory demand increases.

Fully reduced cytochrome oxidase combines very rapidly with oxygen to form an oxygenated intermediate (Chance et al., 1975; Verkhovsky et al., 1996). Equilibrium binding of oxygen in this complex is weak and reversible at room temperature, but operational irreversibility is achieved by kinetic trapping, i.e. fast electron transfer to the oxygen-bound center (Chance et al., 1975; Verkhovsky et al., 1996). The operational $K_m$ for oxygen (the concentration yielding half-maximal steady-state turnover) is not a constant but rather is affected by many parameters and is linearly related to the flux of electrons through the system (Chance, 1965). The operational $K_m$ in state III pigeon (*Columba livia*) heart mitochondria, supplied with oxygen from oxymyoglobin or other oxygenated heme proteins, is close to 0.09 μmol $l^{-1}$ at 25°C [equivalent to 0053 kPa (0.04 torr) oxygen pressure; J.B.W. and B.A.W., unpublished]. Competition between nitric oxide and oxygen for binding to the heme $a_3/Cu_B$ reaction center of cytochrome oxidase will tend to increase the effective operational $K_m$ in the heart from 0.09 μmol $l^{-1}$ to a value within the range of myoglobin-buffered oxygen pressures obtaining in the sarcoplasm (Moncada and Erusalimsky, 2002). *In vivo*,

sarcoplasmic oxygen pressure may, in part, control the rate of reaction of cytochrome oxidase with oxygen.

**Oxymyoglobin controls oxygen utilization and supply**

By acting as a scavenger of the bioactive molecule NO, oxymyoglobin regulates both oxygen supply and utilization. NO is generated continuously in the myocyte. Oxymyoglobin reacts with NO to form the innocuous product nitrate, with concomitant formation of ferric myoglobin, which is recycled through the action of intracellular metmyoglobin reductase. Sarcoplasmic NO concentration is determined by the balance between the rates of these two processes.

The interaction of NO and oxymyoglobin to control cardiac oxygen utilization is demonstrated dramatically in a study by Flogel et al. (2001) of the myoglobin-knockout mouse. Firstly, they demonstrated using NMR spectroscopy that infused NO actually converted oxymyoglobin to ferric myoglobin in the surviving heart. They next demonstrated that infusion of NO, or of bradykinin to stimulate endogenous NO formation, brings about a dramatic fall of coronary perfusion pressure in hearts lacking myoglobin; myoglobin-containing hearts from wild-type mice were little affected. The clear explanation is that oxymyoglobin in the wild-type heart scavenges NO, a powerful vasodilator that increases blood flow and the number of open capillaries.

At higher concentrations of infused NO, cardiac contractility and high-energy phosphate reserves were severely affected by NO in hearts isolated from mice lacking myoglobin and, less so, in hearts from wild-type animals (Flogel et al., 2001). As already noted, NO is a potent but reversible inhibitor of cytochrome oxidase (Moncada and Erusalimsky, 2002). The probable explanation of the results of Flogel et al. (2001), as pointed out by Brunori (2001b), is that intracellular oxymyoglobin, when present, continuously removes NO, thus relieving inhibition of cytochrome oxidase.

The magnitude of the protective effect of oxymyoglobin on cytochrome oxidase activity was demonstrated in an experiment using isolated heart cells held at high oxygen pressures that are sufficient to fully oxygenate intracellular myoglobin. In this condition, oxygen availability does not limit respiratory rate, and myoglobin-facilitated oxygen diffusion contributes no additional oxygen flux. Progressive conversion of intracellular oxymyoglobin to carbon monoxide myoglobin (MbCO) now abolishes about one-third of the oxygen consumption (Fig. 3A). The oxymyoglobin-dependent portion of the oxygen uptake (defined in the legend to Fig. 3A) decreases linearly with increasing mole fraction of intracellular MbCO (Fig. 3B). The probable explanation (Brunori, 2001a) is that intracellular oxymyoglobin continuously removes NO, a reversible inhibitor of cytochrome oxidase. In accordance with the results of Flogel et al. (2001), the effect is large, and about one-third of the total oxygen flux is dependent on oxymyoglobin-mediated dioxygenation of NO. The histological location and the isoform identity of the nitric oxide synthase forming the NO that controls cardiac respiration

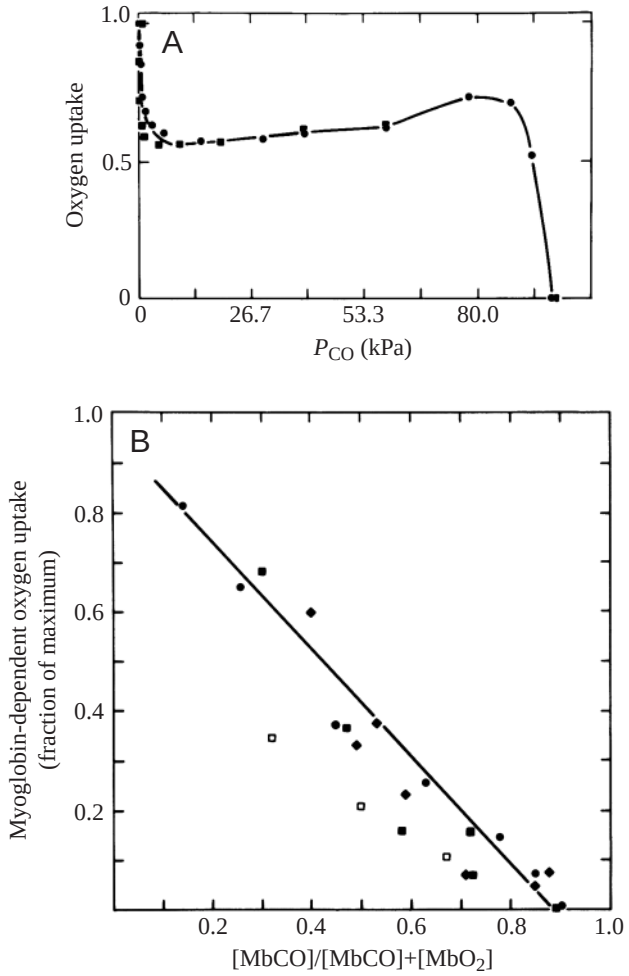


Fig. 3. (A) Steady-state oxygen uptake of suspensions of cardiac myocytes as functions of carbon monoxide partial pressure. Oxygen uptake is normalized, taking the uninhibited rate in each experiment as unity. Oxygen partial pressure ($P_{O_2}$) is equal to 10.6–12.0 kPa (80–90 torr) or 13.3–16.0 kPa (100–120 torr). The oxymyoglobin-dependent portion of the oxygen uptake is taken as the difference between the uninhibited rate and the plateau value at high carbon monoxide partial pressure ($P_{CO}$). Carbon monoxide inhibition of cytochrome oxidase becomes evident above $P_{CO}$=80 kPa (600 torr). Reproduced from Wittenberg and Wittenberg (1987). (B) Oxymyoglobin-dependent oxygen uptake of suspensions of cardiac myocytes as functions of mole fraction carbon monoxide myoglobin (MbCO). Since myoglobin (Mb) is essentially fully occupied by ligands, mole fraction $MbO_2$ = 1 – mole fraction MbCO. In different experiments, $P_{O_2}$ is equal to 5.3–8.0 kPa (40–60 torr), 9.3–12.0 kPa (70–90 torr), 13.3–16.0 kPa (100–120 torr) or 45 kPa (340 torr). Reproduced from Wittenberg and Wittenberg (1987).

remain matters of vigorous controversy (Loke et al., 1999; Kanai et al., 2001; Moncada and Erusalimsky, 2002).

These effects link intracellular oxymyoglobin, oxygen uptake by cytochrome oxidase, capillary oxygen delivery and intracellular NO generation into an integral controlled system. Any transient decrement in oxymyoglobin concentration will be countered by increased sarcoplasmic NO, increased oxygen

input from the capillaries and decreased cytochrome oxidase activity, each tending to restore the initial oxymyoglobin level.

Pearce et al. (2002) challenge the concept that oxymyoglobin regulates the level of NO in cardiac muscle. They present evidence that NO endogenously generated in heart tissues is catabolized with formation of neither nitrate nor ferric myoglobin, the expected products of the reaction of NO with oxymyoglobin. Instead, they find that NO is converted essentially quantitatively to nitrite. They explain this on the basis of a plausible three-electron reduction of NO, catalyzed by cytochrome oxidase. On this basis, they propose that cytochrome oxidase is the major route by which NO is removed from mitochondria-rich cells. It must be emphasized that there is absolutely no conflict between the data sets of Pearce et al. (2002) and those of Flogel et al. (2001) when the experiments were performed under the same conditions.

## Towards a model for oxygen flow in muscle

Drawing on the work of many laboratories, we picture the heart cell and contracting red skeletal muscle fibers as nearly, but crucially not quite, equipotential in oxygen. The diffusion path for myoglobin is tortuous, the local concentration of myoglobin may be great, and myoglobin-facilitated oxygen diffusion, which is rapid relative to other parameters of the system, makes the oxygen pressure nearly the same everywhere in the cell. The largest part of the oxygen pressure drop from the capillary erythrocyte to the mitochondrion occurs outside the cell across the capillary wall. Within the cell, as a consequence of the large area of mitochondrial surface available for diffusion, gradients of oxygen pressure are shallow and the oxygen pressure experienced by cytochrome oxidase closely reflects volume-average sarcoplasmic oxygen pressure.

Muscle and heart, despite access to almost unlimited oxygen, operate in steady states close to the oxygen pressure (0.33 kPa=2.5 torr) required for half-saturation of sarcoplasmic myoglobin with oxygen. In this steady state, the rate of oxygen utilization by cytochrome oxidase, the flux of oxygen across the sarcoplasm and the rate of oxygen entry into the muscle cell must all be the same. Sarcoplasmic oxygen pressure, we suggest, is a controlled parameter and resists change in response to changing workload or oxygen supply. Sarcoplasmic oxygen pressure, together with other parameters, including the concentration of cytochrome oxidase, the fraction of cytochrome oxidase molecules fully reduced and the operational $K_m$ of cytochrome oxidase for oxygen, determine the rate of oxygen utilization. Oxymyoglobin, by destroying NO, limits the blood flow supplying oxygen to the myocyte and relieves NO inhibition of mitochondrial oxygen utilization.

We dedicate this manuscript to the memory of Jeffries Wyman, who first recognized that myoglobin serves as a mobile carrier of oxygen. We thank Dr David Mauzerall and Dr Robert K. Josephson for helpful discussions.

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