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INTRACELLULAR OXYGEN DIFFUSION: THE ROLES OF MYOGLOBIN AND LIPID AT COLD BODY TEMPERATURE

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Summary

Cold temperature can constrain the rate of oxygen movement through muscle cells of ectothermic animals because the kinetic energy of the solvent–solute system decreases and the viscosity of the aqueous cytoplasm increases during cooling within the physiological range of body temperatures. These factors affect the movement of both dissolved oxygen and oxymyoglobin, the two predominant routes of intracellular oxygen diffusion in vertebrate oxidative muscles. In addition, reductions in temperature have been shown to increase the affinity of myoglobin for oxygen and to slow the rate of Mb O_2-dissociation, compromising the ability of this oxygen-binding protein to facilitate intracellular oxygen diffusion. Experiments with both seasonally cold-bodied fishes and polar fish species suggest that several factors combine to overcome these limitations in delivery of oxygen from the blood to the mitochondria. First, reductions in body temperature induce increases in mitochondrial density of oxidative muscle cells, reducing the mean diffusional pathlength for oxygen between capillaries and mitochondria. Second, cold body temperature in both temperate-zone and polar fishes is frequently correlated with a high content of neutral lipid in oxidative muscles, providing an enhanced diffusional pathway for oxygen through the tissue. Third, recent data indicate that myoglobins from fish species bind and release oxygen more rapidly at cold temperature than do those from mammals. Data from both oxidative skeletal muscle and cardiac muscle of fishes suggest that these factors in various combinations contribute to enhance the aerobically supported mechanical performance of the tissues at cold cellular temperatures.

Key words: oxygen diffusion, myoglobin, mitochondria, lipid content, cold temperature, fish muscle.

Cold body temperature and the pathways of intracellular oxygen movement

From a mechanistic standpoint, there are essentially two major routes for the intracellular movement of oxygen in animal cells: (1) physical solution in the solvent system(s) of the cell, and (2) bound as a dissociatable ligand to an oxygen-carrying macromolecule. In the case of physical solution, the solubility of the gas in the aqueous milieu of the cell has, until recently, been considered almost exclusively. For oxidative muscle cells of vertebrate animals (heart and aerobic skeletal muscle), the intracellular oxygen-binding protein myoglobin (Mb), which gives these tissues their characteristic red color, is the oxygen carrier. Transcellular movement of oxygen from capillaries to mitochondria *via* either of these pathways occurs by molecular diffusion and, consequently, can be profoundly affected by cellular temperature. Constraints in the movement of oxygen are most pronounced at the cold body temperatures experienced by many ectothermic animals either seasonally or, in the case of polar species, chronically throughout their life histories. Yet, there exist numerous examples of ectotherms that maintain aerobically supported muscular activity either during cold winter months or in the polar seas of the globe which support abundant and successful populations of fishes and invertebrates. In light of their cold body temperatures, these species clearly must possess mechanisms to ensure a continued supply of oxygen to the mitochondria, where sustained aerobic metabolic power is generated.

In the present paper, I will describe some of the anatomical, physiological and biochemical mechanisms of compensation which have come to light through experiments in this and other laboratories in recent years. I will begin by describing the nature of the constraints in intracellular oxygen movement that are caused by cold cell temperature. In subsequent sections, the suite of compensatory adaptations displayed by cold-bodied animals to overcome these constraints will be outlined.

Oxygen dissolved in the aqueous cytoplasm

The rate of movement of oxygen ($\delta_{O_2}/\delta t$) across muscle tissues under both steady-state (Kawashiro *et al.* 1975) and non-steady-state conditions (Mahler, 1978) has been shown to conform to the one-dimensional diffusion equation for gases as originally modified by Krogh (1919):

$$\delta_{O_2}/\delta t = -K_{O_2} \times A \times (\delta P_{O_2}/x),$$

where K_{O_2} is the diffusion constant for oxygen, which is the numerical product of the diffusion coefficient for oxygen (D_{O_2}) and its solubility coefficient (α_{O_2}), A is the surface area through which the exchange occurs, δP_{O_2} is the partial pressure gradient for oxygen across the diffusion path and x is the length of the diffusion path.

Changing temperature affects both the solubility (α_{O_2}) and diffusion coefficient (D_{O_2}) terms in this expression. D_{O_2} in water increases by approximately 3 % for each rise in temperature of 1 °C. In accord with Henry's Law, however, the solubility of oxygen decreases with increasing temperature by approximately 1.4 % °C^{-1}. The thermal sensitivity of the diffusion constant (K_{O_2}) is therefore approximately 1.6 % °C^{-1}, less than that of the diffusion coefficient alone, but nonetheless quite significant within the physiological temperature range of aquatic ectotherms. For example, cytoplasmic K_{O_2} at the body temperature of an Antarctic fish (−1.8 °C) would be expected to be more than 42 % lower (Q_{10}≈1.2) than that of a temperate-zone fish at a summer temperature approaching 25 °C.

The reduction in D_{O_2} in the aqueous cytoplasm of cells with decreasing temperature is attributable to two factors: (1) a reduction in the kinetic energy of the system (a reduction of less than 7 % on the absolute temperature scale encompasses the entire physiological temperature range of aquatic ectotherms) and, (2) an increase in cytoplasmic viscosity, which is inversely related to cell temperature. Of the two, the viscosity of the aqueous cytoplasm probably plays the more significant role. Indeed, the importance of cytoplasmic viscosity as a determinant of intracellular diffusion has been demonstrated by spin-label (Gershon *et al.* 1985) and other physical techniques (Wojcieszyn *et al.* 1981).

The effect of temperature on the viscosity of aqueous solutions is considerable. Between 25 and 5 °C, a thermal range that includes the physiological temperature range of many temperate-zone fish species, the viscosity of pure water increases by more than 70 %, from 0.89 to 1.52 cP (Weast, 1971). Because the cytoplasm of muscle cells is essentially a polyelectrolytic aqueous solution, we reasoned that it should be characterized by thermally induced viscosity changes of a similar magnitude. Using an undiluted cytosolic preparation from glycolytic muscle tissue of white perch *Morone americanus*, we were able to demonstrate *in vitro* that the kinematic viscosity of the fluid increased from 2.94×10^{-2} to 5.35×10^{-2} cm^2 s^{-1} between 25 and 5 °C, a percentage change only slightly greater than that of pure water (Sidell and Hazel, 1987). The calculated Q_{10} for this change is 1.35±0.01, very close to the Q_{10} of approximately 1.3 between 0 and 22.8 °C determined for oxygen movement through the muscle tissue of frog (Mahler, 1978). Thus, diffusion of oxygen dissolved in the aqueous compartment of the cell appears to be quite sensitive to decreasing temperature, primarily because of a pronounced reduction in D_{O_2} mediated by an elevation in cytoplasmic viscosity.

Oxygen bound to myoglobin

Myoglobin is a monomeric protein of approximately 16 kDa which is capable of binding oxygen at a molecular stoichiometry of 1:1 at its coordinated heme group. Mb is generally abundant in oxidative muscles (both cardiac and skeletal) of vertebrates and its presence is a usual diagnostic feature of these tissues. Unlike hemoglobin, Mb shows no cooperativity and its saturation curve is hyperbolic rather than sigmoidal in nature because it is monomeric. The P_{50} value of Mb (partial pressure of oxygen at which the protein is half-saturated) is very low (⩽0.005 atm or 0.5 kPa), indicating its high affinity for O_2. When saturated with oxygen, Mb is thus capable of binding and removing from physical solution an amount of the gas that is equivalent to its own intracellular concentration. Considerable quantities of data have been accumulated to substantiate the roles of Mb both as an intracellular reservoir and as a facilitator of oxygen diffusion in animal tissues (reviewed by Wittenberg, 1970; Wittenberg and Wittenberg, 1989). Because of its high affinity for O_2, however, it releases bound oxygen only at the very low intracellular partial pressures that would occur in the immediate vicinity of respiring mitochondria or under conditions of environmentally or exercise-induced hypoxia.

Like dissolved oxygen, myoglobin's function as a facilitator of oxygen diffusion should be compromised by the increase in cytoplasmic viscosity that occurs during reductions in cell temperature. Increasing viscosity will negatively affect the diffusion of oxyMb as predicted by the Stokes–Einstein model, which shows that diffusivity is inversely proportional to viscosity (Ried *et al.* 1977). Several other features of Mb oxygen-binding and dissociation make the function of this carrier, at least potentially, even more susceptible to temperature change than is predicted by its diffusivity alone.

High-resolution structural studies (Takano, 1977; Phillips, 1980) and studies of the dynamics of the protein (Lambright *et al.* 1994) have indicated an apparent lack of a static diffusive channel for oxygen through the protein to the heme group. It thus appears that oxygen binding and release from the heme pocket is dependent upon transient pathways that open as a consequence of flexing of the globin structure, a feature that should be strongly affected by changing temperature (Somero, 1995). The lowered structural flexibility of Mb at cold temperatures may be the underlying factor accounting for the considerable decrease in both oxygen dissociation and binding rate constants for Mb from mammalian tissues encountered at cold temperatures (Sato *et al.* 1990; Cashon *et al.* 1997). In fact, it has been argued that the dramatic thermal sensitivity of kinetic constants for oxygen binding and dissociation to Mb are a significant advantage of being warm-bodied (Stevens, 1982; Stevens and Carey, 1981). Thus, the second major route of intracellular oxygen transfer, Mb-mediated facilitated diffusion, will be negatively affected by decreasing temperature because of the reduced molecular diffusion of Mb and MbO_2 caused by increased cytoplasmic viscosity and because of the pronounced slowing of both the binding and dissociation of O_2 and Mb caused by the constrained flexibility of the globin moiety of the protein. In combination, these features suggest that the function of Mb as a facilitator of

oxygen diffusion should be very susceptible to lowering of cell temperature.

The considerations described above indicate that both major routes of intracellular oxygen movement, oxygen in physical solution and bound to Mb, should be severely compromised at cold body temperature in the absence of specific adaptive mechanisms that overcome these constraints. The simplest solution, lowering of cytoplasmic viscosity, is not an available option because of the intrinsic properties of water and can be eliminated from consideration. We will now turn to describing those mechanisms that have been found in animals adapted to cold temperature.

High mitochondrial density is a common ultrastructural feature of animals seasonally or chronically adapted to cold temperature

Classical thermal acclimation studies of eurythermal fishes clearly showed that cold acclimation causes initial decreases in the rates of aerobic metabolism that are followed by a gradual increase in respiration over a period of weeks. As acclimation studies turned to more biochemical indices of metabolic function, it soon became evident that the activities of enzymes from aerobic energy metabolism in highly oxidative tissues increased with cold acclimation in a fashion that appeared to be causally linked to more organismal measures of respiration rate (reviewed by Hazel and Prosser, 1974). During the early 1980s, when quantitative stereological methodologies were applied to ultrastructural studies, it became clear that thermal acclimation of fishes was capable of inducing very dramatic changes in subcellular anatomy that provided an important framework for understanding results from earlier respirometric and enzymatic investigations.

Johnston and Maitland (1980) presented one of the first reports that acclimation to cold temperature altered mitochondrial populations in oxidative muscle of eurythermal fishes. They found that, during acclimation of crucian carp from 28 to 2 °C, the mitochondrial density of red muscle fibers increased by 80 %. During the following several years, similar cold-induced proliferation of mitochondria in oxidative muscle was documented for numerous other fish species, including such phyletically distant animals as goldfish (Tyler and Sidell, 1984), European eel (Egginton and Johnston, 1984) and striped bass (Egginton and Sidell, 1989). The profound nature of this cold-induced mitochondrial proliferation is evident in micrographs of red muscle fibers from 25 °C- and 5 °C-acclimated striped bass, where mitochondria displace 28.6 % and 44.8 % of cell volume, respectively (Fig. 1; Egginton and Sidell, 1989). The trend of an inverse relationship between habitat temperature and the percentage of cell volume displaced by mitochondria was further strengthened by the observation of a parallel relationship in stenothermal fishes from disparate thermal habitats (reviewed by Johnston, 1981). The very high mitochondrial volume densities observed in oxidative muscles of Antarctic fishes, more than 35 % of cell volume (Fitch *et al.* 1984; Londraville and Sidell, 1990*a*,*b*), suggested that high mitochondrial populations were a fundamental requirement for aerobic muscle function at cold temperature, and several putatively adaptive interpretations of this characteristic were articulated.

The most widely recognized potential benefit of increased mitochondrial density at cold cell temperature was an elevation in the concentration of enzymes associated with pathways of aerobic metabolism. Such an elevation in cellular enzyme concentration would compensate for the direct depressing effect of lowered temperature on the catalytic rate of each individual enzyme and would therefore elevate the cell's aggregate metabolic potential. The second, and initially less obvious, benefit was that proliferation of the cell's mitochondrial population reduces mean diffusional pathlength both for the water-soluble small metabolites that exchange between the cytoplasmic and the mitochondrial compartments of the cell and for the oxygen that originates in adjacent capillaries and must travel to the cell's mitochondria. In other words, mitochondrial proliferation at cold temperature can reduce the '*x*' term for oxygen delivery in the one-dimensional diffusion equation, at least partially offsetting the reduction in K_{O_2} because of decreased D_{O_2} through the viscous cytoplasm. As will be discussed below, this is not the only putative benefit derived from the proliferation of this population of organelles.

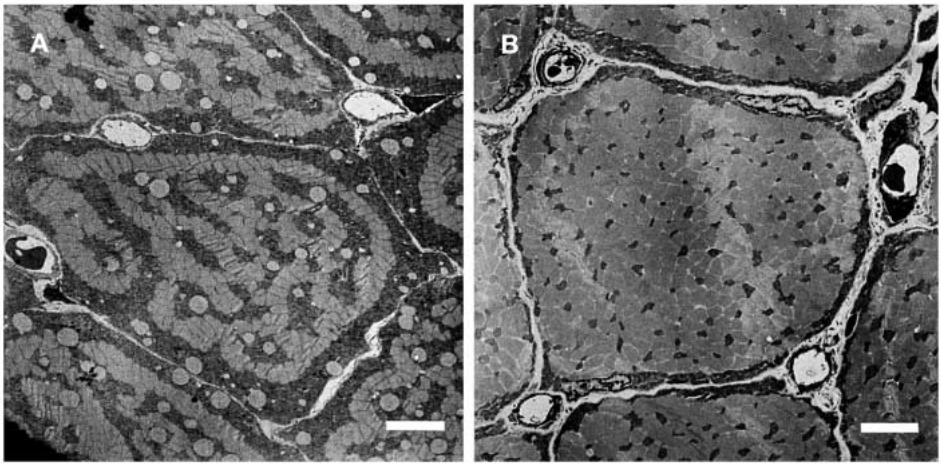

Fig. 1. Electron micrographs of oxidative muscle fibers from axial muscle of striped bass *Morone saxatilis* acclimated to 5 °C (A) and 25 °C (B). Scale bars, 5 μm. Micrograph reproduced with permission from Egginton and Sidell (1989).

A role for cellular lipid in enhancing oxygen movement

Two additional ultrastructural changes caused by acclimation to cold temperature are noteworthy. The first of these is the very striking increase in the content of intracellular neutral lipid droplets (Fig. 1). The percentage of oxidative muscle cell volume of striped bass that is displaced by anatomically discrete neutral lipid droplets increases from 0.6 to 7.9 % between acclimation temperatures of 25 and 5 °C (Fig. 1). Thus, after cold adaptation, almost 10 % of the cell's volume is occupied by a non-polar solvent system with physicochemical characteristics that are vastly different from the aqueous cytoplasm, a situation that must carry some physiological consequence. The second feature, which is initially perplexing, is that populations of mitochondria in cold-adapted animals are often so densely clustered that it is difficult to envision how oxygen might pass to those organelles deep within the cluster. Yet, if mitochondria in the center of these clusters are not adequately supplied with oxygen, their functional significance would seem obscure. As we considered the fundamental physical and chemical characteristics of the cell's constituents, a unifying explanation for both of these observations emerged.

It has long been known that oxygen readily penetrates cells because of its ability to dissolve in the hydrocarbon core of the cell membrane. In fact, oxygen was classified as a fat-soluble molecule that could pass unimpeded through the mesothelial cells of the capillary wall by Pappenheimer (1953) in his classical treatment of capillary permeability. The extent of the affinity of oxygen for nonpolar solvents is demonstrated by its olive oil:water partition coefficient of 4.4 at 25 °C (Battino *et al.* 1968). Furthermore, oxygen solubility in olive oil is almost refractory to temperature change, varying by less than 1 % over a range of 30 °C (Battino *et al.* 1968). Because olive oil is 85 % mixed glycerides of oleic acid, it is a reasonable approximation of fish oils. The relatively large amount of dissolved solutes in the aqueous cytoplasm also will reduce gaseous solubilities, suggesting that the ultimate lipid:cytoplasm partition coefficient may be even higher than that for olive oil:water. Thus, pure physical chemistry suggests that the nonpolar hydrocarbon phase of the cell should contain at least 3–5 times the amount of oxygen that would be contained in an equivalent volume of cytoplasm. In the muscle cells of cold-acclimated animals, the two primary locations of this oxygen-rich lipid phase are neutral lipid droplets (if prevalent) and the hydrocarbon cores of intracellular membrane systems.

By virtue of their large capacity to dissolve oxygen, the neutral lipid droplets found in oxidative muscle cells of cold-bodied fishes have the potential to act both as oxygen reservoirs and as low-resistance elements in the intracellular diffusion pathway for oxygen. We initially reasoned that the high α_{O_2} of the lipid phase could elevate the rate of oxygen movement through tissue from cold-acclimated compared with warm-acclimated animals (Egginton and Sidell, 1989), a concept recently reinforced by the theoretical treatment of Dutta and Popel (1995).

During the past several years, our laboratory has succeeded in empirically testing the hypothesis that a high cellular lipid content enhances the rate of oxygen diffusion through skeletal muscle tissue. We were able to modify methodologies originally developed by Ellsworth and Pittman (1984) to estimate the diffusion coefficient for oxygen through muscle and by Mahler *et al.* (1985) for measurement of tissue solubility of oxygen. We exploited the ability of cold acclimation of striped bass to alter the lipid content of oxidative muscle tissue (Fig. 1) to test whether accumulation of lipid altered K_{O_2} of the tissue when measured under isothermal conditions *in vitro*. The answer was an unequivocal 'yes'.

Table 1. *Effect of structural changes induced by thermal acclimation upon determinants of oxygen diffusion through aerobic skeletal muscle of striped bass* Morone saxatilis

	Acclimation temperature	
	5 °C (N=4)	25 °C (N=5)
$D_{O_2} \times 10^6$ ($cm^2\,s^{-1}$)	2.57±0.40	2.5±0.18
$\alpha_{O_2} \times 10^2$ ($ml\,O_2\,cm^{-3}\,atm^{-1}$)	6.64±0.27	3.59±0.20*
$K_{O_2} \times 10^6$ ($ml\,O_2\,cm^{-1}\,min^{-1}\,atm^{-1}$)	10.4±1.86	5.35±0.40

Values are means ± S.E.M. and were measured at an experimental temperature of 15 °C; N is the number of animals.

Values reported for K_{O_2} are calculated from data where α_{O_2} and D_{O_2} were obtained from the same individual. Because K_{O_2} is a calculated value, statistical comparisons between treatment groups were not performed. An asterisk indicated a significant difference between treatment groups *P<0.001.

Data are from Desaulniers *et al.* (1996).

D_{O_2}, diffusion coefficient for oxygen; α_{O_2}, solubility coefficient for oxygen; K_{O_2}, diffusion constant for oxygen.

1 atm = 101.3 kPa.

Under identical experimental conditions, there was no significant difference between tissues from 25 °C- and 5 °C-acclimated animals in D_{O_2}, but α_{O_2} of the tissue from cold-acclimated fish was almost twofold higher than from warm-acclimated animals (Table 1; Desaulniers *et al.* 1996). Thus, K_{O_2} ($D_{O_2} \times \alpha_{O_2}$), the operative term in describing oxygen diffusion through tissue, is substantially higher in the lipid-rich muscle of cold-acclimated animals, primarily because of its higher oxygen solubility. The correlation between K_{O_2} and lipid content is compelling (Fig. 2).

The same physical chemistry that is involved in the enhancement of oxygen movement described above for lipid droplets should also apply to membranous systems. This theoretical consideration was first presented in an unheralded paper by Longmuir (1980), who suggested that 'oxygen is transported from blood to mitochondria along channels of high solubility' within the cell. Although receiving relatively little credit for this concept, Longmuir's idea is supported by considerable experimental evidence. Using electron spin resonance (ESR) spin-exchange measurements with phosphatidylcholine bilayers, Windrem and Plachy (1980)

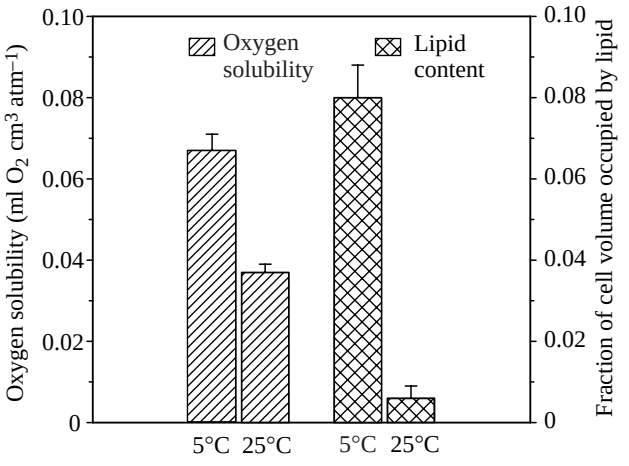

Fig. 2. Relationship between oxygen solubility and content of neutral lipid droplets in oxidative muscle tissue from striped bass *Morone saxatilis* acclimated to 5 °C and 25 °C. Figure is adapted from Desaulniers *et al.* (1996). Values are means + S.D. (*N*=4).

have shown that K_{O_2} is substantially greater in the hydrocarbon core of the bilayer than near the polar head groups. Subczynski *et al.* (1989, 1991) have also used spin-label methodologies to show that the major resistance to oxygen transport in membranes is at the polar head group of the phospholipids, and that oxygen transport in membranes is affected by both the degree of alkyl chain saturation and intercalation of cholesterol molecules into the membrane. This, combined with evidence that the membrane:water partition coefficient for oxygen is significantly greater above the phase-transition temperature (Smotkin *et al.* 1991), has led Dutta and Popel (1995) to theorize that, in their normal phase state, "membrane lipids (are) the preferred pathway for intracellular oxygen transport over the relatively 'slow' aqueous cytoplasm phase". Results showing that oxygen movement through the hydrocarbon core of biological membranes is affected by the degree of akyl chain saturation and decreased below critical phase transition temperatures or by the addition of membrane-rigidifying insertions of cholesterol (Subczynski *et al.* 1989, 1991; Smotkin *et al.* 1991) further suggests a previously unappreciated benefit of the process of homeoviscous adaptation of membranes to temperature change.

Upon exposure of ectothermic animals to cold temperature, the hydrocarbon chains of membrane phospholipids become significantly more unsaturated. This alteration in membrane composition has been interpreted as being adaptively significant in ensuring that biological membranes maintain an unaltered degree of fluidity or microviscosity at cold temperatures that could otherwise threaten a significant rigidifying of previously more saturated phospholipid acyl chains. The maintenance of biological membranes in a relatively constant state of fluidity would allow unimpeded conformational flexibility of important membrane-bound enzymes and ion channels that are necessary for normal cellular function; this putatively adaptive compensation to temperature change has been termed 'homeoviscous adaptation' (reviewed by Hazel, 1995). On the basis of the considerations described above, it is equally clear that these same mechanisms that maintain the fluidity of the hydrocarbon core should also keep cellular membranous systems patent for rapid movement of oxygen.

The combined weight of both theoretical treatments and experimental evidence strongly argues that intracellular membranous systems form a lacy anastomosing network of rapid transport pathways for oxygen through the eukaryotic cell. The proliferation of mitochondria at cold body temperatures can be viewed as multiplying the system of channels for the rapid delivery of oxygen. These membranous conduits for oxygen delivery also provide an explanation of how mitochondria in the center of clusters, where mitochondrial membranes are often in close juxtaposition, are able to obtain oxygen to support respiratory function.

A role for myoglobin at cold body temperature

Earlier in this essay, several factors were considered that would seem to mitigate against normal function of the intracellular oxygen-binding protein, myoglobin, in meeting physiological roles as a reservoir and facilitator of oxygen diffusion at cold body temperature. To be fair, I should now point out that most of these arguments were based upon available evidence for the effect of temperature on the best-studied myoglobin molecules, almost exclusively from mammals. The evidence, however, was sufficiently strong that, as I and my coworkers began a few years ago to investigate the possible physiological relevance of myoglobin in tissues of chronically cold-bodied Antarctic fishes, my best guess was that we would find a diminished importance of the hemoprotein in the oxygen economy of their tissues. Our accumulating data have shown that, alas, my instincts were wrong.

Our recent work has focused on the unique hemoglobinless family of Antarctic icefishes, the Channichthyidae. In addition to lacking circulating hemoglobin and red blood cells (reviewed by Hemmingsen, 1991), the consensus in the literature until recently has been that these fishes also do not express intracellular Mb in their tissues. Using immunochemical and molecular techniques, we have recently reported that Mb is expressed by several (but not all) species within the icefish family but, when present, is found exclusively in cardiac muscle (Sidell *et al.* 1997). When present, Mb is found in concentrations within the range of those reported for temperate-zone fishes (T. J. Moylan and B. D. Sidell, in preparation). The closely related Mb-expressing and Mb-lacking species of Antarctic icefishes thus provide an excellent natural experimental system for isolating the physiological consequences of the presence and absence of Mb in a working oxidative muscle from a group of animals that has been evolving at a body temperature of approximately 0 °C for the last 15–25 million years. We have been approaching this question using the combined tools of biochemistry, physiology and molecular biology.

The family of hemoglobinless Antarctic icefishes

Table 2. *Pattern of expression of cardiac myoglobin among species of the Antarctic icefishes* Channichthyidae

Genus	Species	Myoglobin concentration (mg g^{-1} wet mass)
Chionodraco	*rastrospinosus* (*N*=6)	0.64±0.07
	hamatus (*N*=6)	0.62±0.04
	myersi (*N*=4)	0.71±0.08
Pseudochaenichthys	*georgianus* (*N*=6)	0.46±0.04
Cryodraco	*antarcticus* (*N*=6)	0.44±0.02
Chaenodraco	*wilsoni* (*N*=6)	0.73±0.12
Chionobathyscus	*dewitti* (*N*=2)	0.70±0.02
Neopagetopsis	*ionah* (*N*=1)	0.70
Chaenocephalus	*aceratus* (*N*=6)	ND
Pagetopsis	*macropterus* (*N*=1)	ND
	maculatus (*N*=1)	ND
Champsocephalus	*gunnari* (*N*=6)	ND
Dacodraco	*hunteri* (*N*=4)	ND

All data are mean ± S.E.M. for the number of animals shown in parentheses.

Data are from T. J. Moylan and B. D. Sidell (in preparation).

ND, not detected; Mb, myoglobin.

Channichthyidae contains 15 known species and 11 genera. To date, we have been able to establish the pattern of Mb expression in 13 of those species representing 10 genera (Table 2). We have used antibodies specifically raised against Mb to show that the protein is found in the heart ventricles of eight species, but absent from the same tissue in five species that are widely dispersed among clades of the accepted phylogeny of the family (Sidell *et al.* 1997; T. J. Moylan and B. D. Sidell, in preparation). The most parsimonious interpretations of this pattern seemed to be that Mb function was sufficiently impeded at the body temperatures of these animals (approximately 0 °C), that the protein was a relic of earlier ancestry but was essentially vestigial and that loss of its expression by any mechanism might be selectively neutral. We have been testing this hypothesis with a multi-pronged experimental effort aimed at determining the functional significance of myoglobin in those species that express the protein.

Oxygen binding kinetics of myoglobins

In collaboration with colleagues Robert Cashon and Michael Vayda, we have compared the effect of temperature on rates of both oxygen binding and dissociation of myoglobins from Antarctic fish species with those from warmer-bodied fishes and mammals (Cashon *et al.* 1997). Our results clearly show that both on-constants for carbon monoxide (considered to mimic oxygen binding, but more experimentally approachable) and oxygen dissociation constants for myoglobins from Antarctic fishes are considerably more rapid than those from mammalian species at all experimental temperatures (Figs 3, 4). Although this characteristic appears not to be unique to

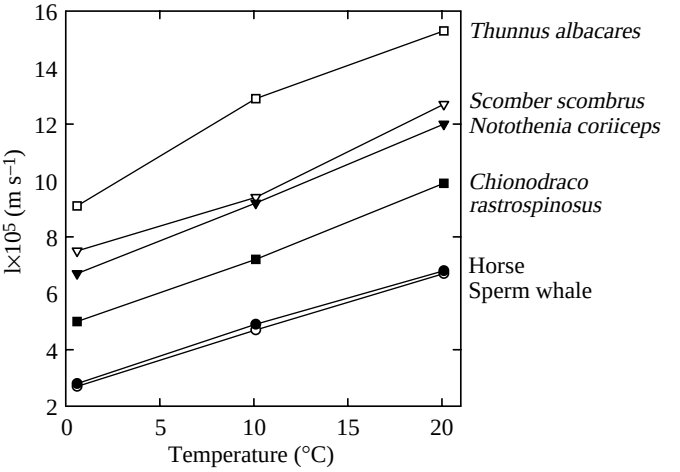

Fig. 3. Thermal sensitivity of the carbon monoxide association constant (*l*) of myoglobins from two mammals (circles), two temperate-zone fishes (open squares and triangles) and two Antarctic fishes (filled squares and triangles). Figure is adapted from Sidell and Vayda (1997); data from Cashon *et al.* (1997).

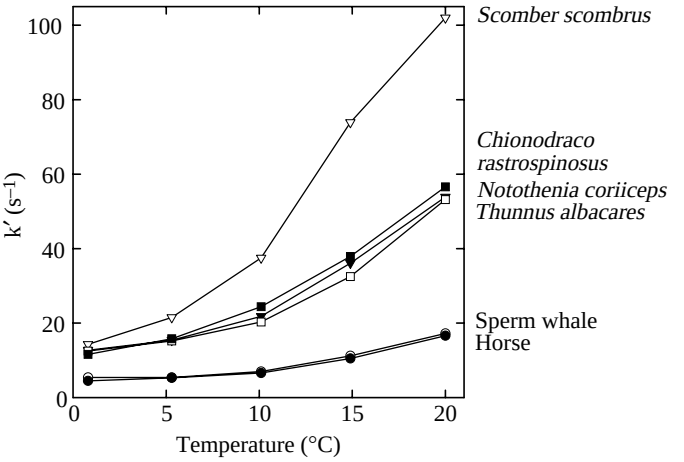

Fig. 4. Thermal sensitivity of the oxygen dissociation constants (*k*′) of myoglobins from two mammals (circles), two temperate-zone fishes (open squares and triangles) and two Antarctic fishes (filled squares and triangles). Figure is adapted from Sidell and Vayda (1997); data from Cashon *et al.* (1997).

Antarctic teleosts compared with warmer-bodied fishes, the functional implications remain clear. At cold body temperature, the Mb of Antarctic fishes should both bind and release oxygen more rapidly than would be predicted from the behavior of the mammalian proteins that are usually studied. Because these processes are dependent upon the structural flexibility of the globin moiety, it seems reasonable to conclude that the Mbs of teleosts are inherently more conformationally open and flexible than those of warm-bodied endotherms, a trend paralleled by many other proteins in these groups (see Somero, 1995). Thus, the oxygen binding kinetics suggests that the myoglobins of fishes should be functional, even at cold body temperature.

Isolated, perfused heart experiments

Experiments conducted in collaboration with colleagues Raffaele Acierno, Claudio Agnisola and Bruno Tota provide even more direct evidence supporting the functional significance of Mb in Antarctic icefishes. We have recently compared, at a physiological temperature of 0 °C, the mechanical performance of isolated, perfused hearts from two channichthyid icefish species, one in which Mb is present in the heart ventricle (*Chionodraco rastrospinosus*) and one that does not produce the protein (*Chaenocephalus aceratus*) (Acierno *et al.* 1997). To minimize the potential of factors other than the presence and absence of myoglobin influencing the results, these species were selected because of their very close phyletic relationship within the *Channichthyidae* (Iwami, 1985) and the similarity of their relatively inactive demersal lifestyles.

When isolated, perfused hearts from *C. rastrospinosus* and *C. aceratus* were subjected to increasing pressure challenges of afterload at physiological levels of filling pressure, hearts from the former Mb-containing species were found to be capable of significantly greater pressure–work than those of the latter species which lack Mb (Fig. 5). Although this result clearly correlates the presence of cardiac Mb with greater mechanical performance, we recognized that such a conclusion was not definitive; other structural or metabolic factors could have contributed to the observed differences in work capabilities between the tissues. To isolate Mb as a causative factor more convincingly, we repeated the experiments incorporating into the perfusion medium 5 mmol l^{-1} $NaNO_2$, which specifically and reversibly poisons Mb. When Mb function is selectively ablated by 5 mmol l^{-1} $NaNO_2$, the mechanical performance of hearts from Mb-containing *C. rastrospinosus* is significantly decremented, while those of *C. aceratus* that do not contain the protein remain refractory to the treatment. In fact, the decrease in mechanical performance of Mb-poisoned hearts from *C. rastrospinosus* is so pronounced that their performance even falls significantly below that of the normally Mb-deficient hearts of *C. aceratus*: the cardiac output of $NaNO_2$-poisoned hearts of *C. rastrospinosus* is reduced to 50 % of initial levels at an afterload challenge of 3.23±0.10 kPa, while those of hearts from Mb-lacking *C. aceratus* are not impaired to this extent until an afterload of 3.49±0.01 kPa (Acierno *et al.* 1997). These results suggest two conclusions: (1) the presence of myoglobin in hearts of Antarctic icefish apparently contributes to enhanced mechanical performance of the tissue under conditions of high work demand, and (2) subsequent to the evolutionary loss of Mb, compensatory mechanisms (structural or metabolic) have developed in hearts of the Mb-deficient species that partially overcome for loss of this protein.

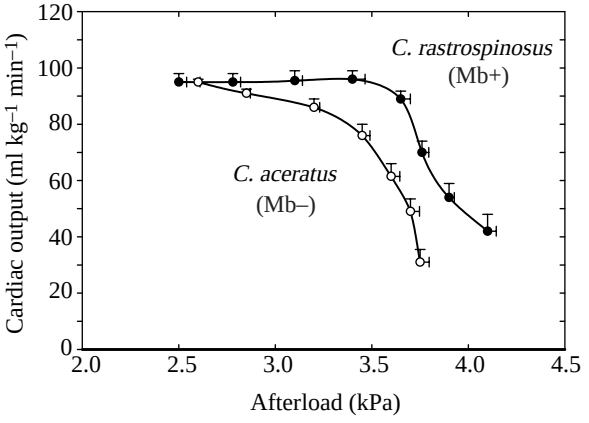

Fig. 5. The ability of isolated, saline-perfused hearts from an icefish species that expresses myoglobin (Mb) (*Chionodraco rastrospinosus*) and from one that lacks Mb (*Chaenocephalus aceratus*) to perform mechanical work in response to afterload pressure challenge. Figure is reproduced from Sidell and Vayda (1997); data are taken from Acierno *et al.* (1997). Values are means + S.D. (*N*=5).

Conservation of the myoglobin gene sequence

The final approach that we have been employing to test for evidence of myoglobin function in the Antarctic icefishes takes advantage of current methodologies in molecular biology. In collaboration with my colleague Michael Vayda and his associates, we have been able to obtain full-length cDNA sequences from several icefish species and from at least two species of a closely related (same suborder) family of red-blooded Antarctic fishes, the Nototheniidae. If Mb is, in fact, a vestigial protein of no functional consequence, then the expectation would be that sequence divergence of the Mb gene among species would be considerable because of relief of selective pressure for maintaining functional protein. Conversely, if Mb function does confer selective advantage, we would expect to see strong conservation of the coding sequence of the gene. Our results strongly support the latter conclusion.

We were able to determine the myoglobin cDNA sequence for seven Antarctic notothenioid fish species; five species were from the channichthyid icefish family and two others from the closely related red-blooded Nototheniidae (Vayda *et al.* 1997). Among the species that express the protein, Mb cDNA sequences were highly conserved; sequence variation was 2.0–2.9 % in the coding region and 2.6–3.3 % over the entire cDNA. Similar sequence variation was observed when comparing the Mb-expressing icefishes with their red-blooded notothenioid relatives. Two icefish species that have lost the ability to produce Mb, however, showed the highest level of sequence variation among the myoglobin genes examined. Subsequent analyses of full-length genomic DNA for the myoglobin gene has further shown that sequences downstream from the polyadenylation site in three species of icefish examined bear no homology, indicating that a rapid sequence change has occurred since the divergence of the species (Small *et al.* 1998). The drift of myoglobin sequence in those species that have lost the ability to express the protein, the significant sequence variation in the non-coding regions of the gene and the marked conservation of sequence in the coding region for the protein all are consistent with the conclusion that selective pressure operates to maintain myoglobin in those species that continue to express the protein.

In combination, the weight of evidence from experiments on oxygen-binding kinetics, the performance of isolated hearts and the gene sequencing described above appears strongly to support the conclusion that Mb is functional at the physiological temperatures of cold-bodied fishes.

Differentiation of the roles of intracellular lipid and myoglobin in oxygen movement at cold temperature

In the previous sections of this essay, I have presented evidence that suggests roles both for intracellular lipid and for myoglobin in the oxygen economy of aerobic muscle cells at cold body temperature. It is reasonable at this point to question whether these simply represent redundant systems for intracellular movement of oxygen or whether their relative roles can be differentiated temporally, spatially or physiologically. Clearly, both lipid and myoglobin have the capacity to serve as intracellular reservoirs for oxygen beyond the amount of O_2 that would otherwise be found in physical solution in the aqueous cytoplasm. Likewise, there is compelling evidence that each can function to enhance the rate of transcellular mass-flux of oxygen at any given P_{O_2} gradient from capillaries to mitochondria. However, significant differences in the characteristics of these systems suggest that their physiological roles can be differentiated.

The underlying mechanisms of oxygen association with intracellular lipid and myoglobin are fundamentally different. In the case of lipid, the gas is present in physical solution in the nonpolar solvent phase. At any point where a P_{O_2} gradient develops between aqueous and lipid phases of the cell (for example, in response to increased respiratory demand), oxygen will flow along this partial pressure gradient between the two solvent phases. Myoglobin, however, binds oxygen as a dissociatable ligand with characteristic affinity and kinetic constants for binding and release. Most importantly, the very high affinity of Mb for O_2 relegates its role exclusively either to areas of the cell with extremely low P_{O_2} or to conditions of environmental or demand-based hypoxia. The significance of these different mechanisms can be illustrated by returning to the example of lipid accumulation at cold temperature by aerobic muscle of striped bass that was cited earlier (see Fig. 1).

By using the measured the lipid and myoglobin content of striped bass muscle and estimates of oxygen solubility in the lipid phase, we have been able to evaluate the amounts of oxygen associated with cellular lipid and myoglobin in animals acclimated to both 25 °C and 5 °C (Desaulniers *et al.* 1996). For the tissue from 25 °C-acclimated fish that contain very little lipid, the picture is not particularly exciting. At any steady-state condition short of near-anoxia, the pool of oxygen bound to Mb is substantially greater than that associated with the lipid phase of the cell (Fig. 6A). However, when a similar analysis is performed for lipid-rich tissue from animals acclimated to 5 °C, a much more interesting picture emerges (Fig. 6B). At any cellular P_{O_2} above approximately 0.035 atm (approximately 3.55 kPa), we project that more oxygen will be found in the lipid phase of the cell than bound to Mb in tissue from cold-acclimated animals! (It is worthwhile pointing out that these calculations are conservatively based on only the amount of lipid in anatomically discrete droplets and do not include consideration of the alkyl chains of intracellular membranes. Inclusion of the latter would cause the slope of the line describing the oxygen content of lipid in Fig. 6B to be even steeper and the P_{O_2} of equivalence in oxygen content between Mb and lipid to be even lower.) Most importantly, any decreases in cellular P_{O_2} will result in a flow of oxygen from the lipid to the aqueous phase of the cell, as described by the dashed line in Fig. 6B. Oxygen associated with Mb, however, will remain bound to the protein throughout most of this range of P_{O_2} values until extremely low partial pressures in the region of the P_{50} value of the protein of approximately 0.001 atm (approximately 0.1 kPa).

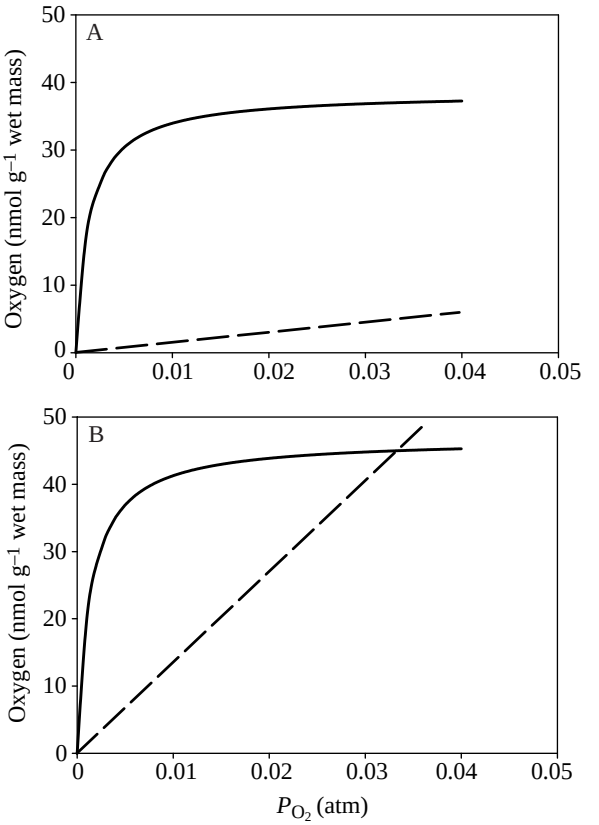

Fig. 6. Estimation of the amount of oxygen associated with intracellular lipid (dashed line) and with myoglobin (solid line) in oxidative muscle of striped bass *Morone saxatilis* acclimated to 25 °C (A) and 5 °C (B) as a function of P_{O_2}. The amount of oxygen dissolved in lipid is based solely upon the volume of anatomically discrete neutral lipid droplets. Figure is adapted from Desaulniers *et al.* (1996). 1 atm = 101.3 kPa.

From the above considerations, we can consider cellular lipid and myoglobin to represent a two-tiered system for ensuring oxygen availability to aerobic muscle at cold body temperatures. Throughout the entire range of physiological P_{O_2} values, intracellular lipid serves both as a reservoir of oxygen

and as an enhancer of oxygen diffusion. These roles of lipid in the oxygen economy of the cell may be particularly important at cold body temperatures when the oxygen demand of aerobic muscles may increase more rapidly than can be matched by cardiovascular adjustments in the delivery of oxygen. Although Mb can also serve similar functions in the cellular microenvironments of locally low oxygen partial pressure, its major role is realized as a back-up system of last resort when cellular P_{O_2} values become extraordinarily low because of increased oxygen demand or other conditions contributing to relatively profound cellular hypoxia.

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