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Myoglobin-enhanced oxygen delivery to isolated cardiac mitochondria

Jonathan B. Wittenberg and Beatrice A. Wittenberg
Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461, USA
*Author for correspondence (e-mail: jwitten@post.harvard.edu)

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Summary

The heart, red skeletal muscles and the nitrogen-fixing legume root nodule function in steady states of high oxygen influx, partial oxygenation of cytoplasmic myoglobin or leghemoglobin and correspondingly low oxygen partial pressure. Here, we ask: what conditions are required at the surface of actively respiring, state III, tightly coupled mitochondria to enhance oxygen flow to cytochrome oxidase? Pigeon heart mitochondria were isolated with minimal damage to the outer mitochondrial membrane and were incubated at low oxygen pressures, where respiration is oxygen limited, with solutions of each of six monomeric hemoglobins with widely divergent kinetics and equilibria in their reactions with oxygen: Busycon myoglobin, horse myoglobin, Lucina hemoglobins I and II, soybean leghemoglobin *c* and Gasterophilus hemoglobin. Each augments oxygen uptake. The declining fractional saturation of each hemoglobin with oxygen was monitored spectrophotometrically as mitochondrial respiration depleted the oxygen; the oxygen partial pressure at half-maximal rate of oxygen uptake was similar for each hemoglobin, supporting the conclusion that the hemoglobins did not interact with the mitochondrial surface in oxygen delivery. The oxygen pressure required to support state III mitochondrial oxygen uptake, 0.005 kPa (0.04 torr), is small compared with that obtained in the sarcoplasm and at the mitochondrial surface of the working heart, 0.32 kPa (2.4 torr). We conclude that, in normal steady states of contraction of the myoglobin-containing heart, oxygen utilization by mitochondrial cytochrome oxidase is not limited by oxygen availability.

Key words: myoglobin, oxygen, facilitated diffusion, heart, muscle, isolated cardiac mitochondria, cytochrome oxidase, pigeon.

Introduction

As a consequence of the limited solubility of oxygen in water, the purely diffusive inflow of oxygen into working muscle and some other rapidly respiring tissues falls short of the demand, and a cytoplasmic oxygen transport system is required to ensure an adequate oxygen inflow. Myoglobin, a mobile carrier of oxygen, is developed adaptively in red muscle in response to mitochondrial demand for oxygen (Millikan, 1939; Wittenberg, 1970; Williams and Neufer, 1996; Yan et al., 2001) and transports oxygen from the sarcolemma to the mitochondria of vertebrate heart and red muscle cells (Wittenberg and Wittenberg, 1989; Wittenberg and Wittenberg, 2003; Sidell and O'Brian, 2006). 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, membrane-bound intracellular organelles housing the bacteroids, the intracellular nitrogen-fixing form of the bacterium *Rhizobium* (Wittenberg et al., 1974).

Because the concentration of myoglobin in cardiac and red skeletal muscle is very great, reaching 3 mmol l^{-1} in the sarcoplasmic domain to which it is confined, it is not surprising that myoglobin serves multiple functions. In addition to its role in oxygen transport, myoglobin, by serving as a sink for nitric oxide (NO), regulates both oxygen inflow into the muscle cell and oxygen consumption by mitochondrial cytochrome oxidase (Wittenberg and Wittenberg, 2003; Mammen et al., 2003; Antunes et al., 2004). Elegant experiments prove that NO-mediated control of oxygen usage plays a major role in the functioning of the intact heart (Flogel et al., 2001; Stumpe et al., 2001; Mammen et al., 2003; Merx et al., 2005) and regulates at least one-third of the oxygen uptake of isolated cardiac myocytes (Wittenberg and Wittenberg, 1987; Wittenberg and Wittenberg, 2003; Mammen et al., 2003). In addition to its functions in oxygen supply and NO regulation, myoglobin serves as an antioxidant defense in the heart (Flogel et al., 2004), may act as a mobile carrier of fatty acids (Gloster and Harris, 1977; Gotz et al., 1994), and is importantly involved in maintaining fatty acids as the main substrate for cardiac metabolism (Flogel et al., 2005).

Krogh (Krogh, 1919) and Wyman (Wyman, 1966), taking simplified assumptions, developed equations solely to exemplify the flow of oxygen in tissue. Recent determinations of the translational diffusion coefficient of myoglobin in heart and muscle have been taken as the occasion to use these equations predictively, leading to the conclusion that the calculated

myoglobin-assisted flux of oxygen into heart and muscle is far less than that actually observed (Jurgens et al., 2000; Lin et al., 2006). Major difficulties in the predictive use of these equations are that the model does not correspond to conditions thought to exist in living muscle (Wittenberg and Wittenberg, 2003) and that the parameters employed can be approximated with only poor precision. Perhaps, however, the discordance between prediction and actuality serves to warn that there is more to be learned about myoglobin function than we already know.

In this study we address the delivery of oxygen from oxymyoglobin to isolated mitochondria in order to define the conditions required at the mitochondrial surface to support oxygen flow to cytochrome oxidase when oxygen supply is limiting. To this end, mitochondria are suspended in solutions of oxygen-binding proteins with widely divergent kinetics and equilibria in their reactions with oxygen, and the rates of state III mitochondrial oxygen uptake are measured as functions of solution oxygen pressure at those low oxygen pressures where oxygen uptake would be oxygen-limited in the absence of carrier protein. The presence of a presumed 'docking site', a planar circle of seven conserved charged residues on the myoglobin surface near the C-D interhelical bend (Romero-Herrera et al., 1978), keeps alive the possibility that myoglobin binds transiently to some protein or surface in the course of its function. We find that myoglobin need not bind to the mitochondrial surface to deliver its oxygen. We also find that, although reversible oxygenation is a prerequisite for oxygen transfer, oxygen affinity and the kinetics of the reactions of each protein with oxygen are not crucial to oxygen delivery to mitochondria. We discover that the sole boundary condition required to support state III mitochondrial oxygen uptake is that the oxygen pressure at the outer mitochondrial membrane exceed a value of approximately 1.3×10^{-3} to 9.3×10^{-3} kPa (mean 0.0053 kPa) (0.01–0.07 mmHg, mean 0.04 mmHg). This is much less than the canonical value of sarcoplasmic oxygen pressure in the heart or muscle operating in a steady state, 0.33 kPa (2.5 mmHg) (Wittenberg and Wittenberg, 2003). We conclude that cytochrome oxidase in the normal heart is not limited by oxygen supply and experiences oxygen pressures near that in the sarcoplasm (see also Wittenberg and Wittenberg, 2003).

Materials and methods

Reagents

Sucrose and mannitol were from Baker (analyzed reagent). Bovine serum albumin (fatty acid-free) was from Boehringer Mannheim. L-malic acid was from Fluka. Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Ultrol grade) was from Calbiochem. ATP, ADP, hexokinase, catalase, superoxide dismutase, heparin and L-glutamic acid were from Sigma.

Optical spectra

These were acquired using a modified Cary model 17 recording spectrophotometer equipped with a temperature-controlled cell holder (Aviv Associates, Lakewood, NJ, USA). This was equipped with an Aviv Associates Scattered Transmission Accessory for use with light-scattering suspensions of mitochondria.

Homogenizer

The homogenizer used for pigeon hearts (~3 g tissue) was modified from a teflon-pestle tissue homogenizer with serrated tipped pestle (Arthur H. Thomas Co., Philadelphia, PA, USA; catalogue #3431-E04, size C) by reducing the diameter of the pestle to give a wide (0.25 mm) clearance between the pestle and the outer glass member. A modified homogenizer, size B, clearance 0.20 mm, was used for rat hearts (~1 g tissue).

Hemoglobin preparation

All hemoglobins were fully oxygenated and contained less than 5% ferric protein. Horse (*Equus caballus* L.) heart myoglobin (Sigma) was reduced anaerobically with sodium dithionite and passed over a Sephadex G-25 column (Pharmacia) to remove excess dithionite and products. *Busycon canaliculatum*, L. (a gastropod mollusc) radular myoglobin was prepared by a standard procedure (Wittenberg and Wittenberg, 1981). *Lucina pectinata*, Gmelin (a bivalve mollusc) hemoglobin I and hemoglobin II were prepared by the method of Kraus and Wittenberg (Kraus and Wittenberg, 1990). Soybean (*Glycine max*, L.), leghemoglobin *c* was prepared by the method of Appleby et al. (Appleby et al., 1975). *Gasterophilu* sp. (a dipteran insect) hemoglobin was prepared by an adaptation of the procedure of Phelps et al. (Phelps et al., 1972), with final purification by chromatography on a Sephadex G-75 column (Pharmacia). All hemoglobins are monomeric at the concentrations used.

Pigeon heart mitochondria

The isolation procedure used was based on those of Toth et al. (Toth et al., 1986) and Berkich et al. (Berkich et al., 1991). A highly purified collagenase from *Clostridium histolyticum* (Sigma type VII) was used. To avoid contact between the active collagenase and the mitochondrial outer membrane, the collagenase was inactivated before disrupting the muscle cells.

Pigeons were euthanased by cervical dislocation following a protocol approved by the Animal Institute Committee of the Albert Einstein College of Medicine. Excised hearts from heparinized adult pigeons (*Columba livia*, L.) were placed in 50 ml of warm (40°C) osmotic support medium and allowed to beat until largely free of blood. The medium contained mannitol (225 mmol l^{-1}), sucrose (75 mmol l^{-1}) and 10 mmol l^{-1} sodium Hepes buffer, pH 7.4. A cannula, fabricated from 12-gauge stainless steel hypodermic tubing and attached to a 100 ml syringe filled with ice-cold osmotic support medium, was placed in the right pulmonary artery, and the heart was submerged in ice-cold osmotic support medium. Residual blood was removed by perfusing approximately 60 ml of medium through the heart. The cannula was transferred to a 10 ml syringe and the heart was perfused slowly with 10 ml of a solution of collagenase (40 units ml^{-1}) in osmotic support

medium. The ventricle was cut free from the cannula and immediately minced with scissors in 10 ml of the ice-cold collagenase solution. After 3 min the mince was washed repeatedly with an ice-cold solution containing ethyleneglycoltetraacetic acid (EGTA) (1.0 mmol l^{-1}) and bovine serum albumin (2 mg ml^{-1}) in osmotic support medium. EGTA, by sequestering calcium, inactivates the metal-dependent added collagenase as well as endogenous proteases. EGTA also moderates mitochondrial calcium uptake (Toth et al., 1986). The mince was transferred to a homogenizer with 50 ml of an ice-cold solution containing EGTA (1.0 mmol l^{-1}), bovine serum albumin (2 mg ml^{-1}) and disodium ATP (2.0 mmol l^{-1}). The mince was homogenized using three strokes of the pestle rotating at 500 rpm, and mitochondria were isolated from the homogenate by differential centrifugation (Toth et al., 1986). The washed mitochondria were suspended in 1.0 ml of osmotic support medium containing EGTA (1.0 mmol l^{-1}) and bovine serum albumin (2 mg ml^{-1}) to give approximately 30 mg mitochondrial protein per ml.

Criteria of mitochondrial integrity

Retention of fatty acyl-coenzyme A synthetase activity and the absence of respiratory stimulation by 2 mmol l^{-1} exogenous ferrous cytochrome *c* together demonstrate that the outer mitochondrial membrane suffered little damage during the isolation procedure (Toth et al., 1986). The mitochondria were tightly coupled, with ADP phosphorylated/atomic oxygen (P/O) ratios of 2.44±0.03 (*N*=24). This compares favorably with the P/O ratio, 2.41, determined non-invasively in the intact perfused rat heart (Kingsley-Hickman et al., 1990). The respiratory control index (RCI), using glutamate plus malate as substrate, always exceeds 6 and is usually greater than 15. The specific activity was 180–220 mol O_2 consumed (mol cytochrome aa_3)$^{-1}$ min^{-1}. This compares favorably with that found by Toth et al. (Toth et al., 1986) and others. The mitochondrial preparation is highly reproducible, enabling comparison of experiments performed with different preparations, as was done here. The specific activities of 29 independent preparations were 195±7 mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1} (mean and s.e.m.).

Cytochrome oxidase (cytochrome aa_3) determination

Mitochondrial pellets are solubilized in a solution containing 1% (w/v) deoxycholate in 50 mmol l^{-1} sodium phosphate buffer, pH 7.8. Potassium phosphate buffer should not be used. The resulting solution was clarified by passage through a 0.45 μm syringe filter (Nalgene), and a difference spectrum, dithionite reduced minus ferricyanide oxidized, was acquired. A value, Δe (605–630 nm)=24 mmol l^{-1} cm^{-1}, was used to calculate the concentration of cytochrome aa_3 (Van Gelder, 1966).

ADP determination

ADP was determined spectrophotometrically, taking e_{259}=15.4 mmol l^{-1} cm^{-1}.

Polarographic determination of mitochondrial oxygen uptake

Oxygen uptake was monitored at 25°C in a completely fluid-filled chamber equipped with an oxygen-sensing electrode (Model 2110; Orion, Geneva, Switzerland), an injection port and a motor-driven stirring paddle. Mitochondria (approximately 2 pmol as cytochrome aa_3) were suspended in a medium containing mannitol (225 mmol l^{-1}), sucrose (75 mmol l^{-1}), glutamic acid (5 mmol l^{-1}), L-malic acid (2.5 mmol l^{-1}) and 10 mmol l^{-1} potassium phosphate buffer, pH 7.2. Oxygen pressure is recorded as a function of time from P_{O_2} of approximately 21 kPa (155 mmHg; air) to approximately 1.3 kPa (10 mmHg); the range within which the electrode current is linear in P_{O_2}. Increments of ADP (250 or 500 nmol) are injected at intervals. The rate of oxygen uptake is expressed as mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1}.

Respiratory parameters

Following Chance and Williams (Chance and Williams, 1956), state III and state IV respiration are defined as mitochondrial respiration in the presence and absence, respectively, of adequate ADP. The specific activity, mol O_2 (cytochrome aa_3)$^{-1}$ min^{-1}, is defined as the rate of state III respiration, with glutamate plus malate as substrate, per unit cytochrome aa_3. The P/O ratio for each increment of added ADP is given by the ratio of ADP consumed to two times the diatomic oxygen consumed. It is calculated from the amount of ADP added, the oxygen partial pressure difference during state III respiration, the volume of the chamber (3.7 ml) and the concentration of oxygen in air-equilibrated medium (257 μmol l^{-1} at 25°C). The respiratory control index (RCI) is the ratio of the rate of state III respiration to the rate of subsequent state IV respiration.

Spectrophotometric determination of mitochondrial oxygen uptake

The commonly used polarographic method of determining mitochondrial oxygen uptake in practice is limited to the range of P_{O_2}, 20–1.3 kPa (155–10 mmHg). The spectrophotometric procedure used here extends the measurements into the region of interest in the physiology of red muscle, say 0.7 to 0.001 kPa (5 to 0.01 mmHg). In this method, the changing fractional saturation of an added myoglobin or hemoglobin is followed spectrophotometrically, and the oxygen uptake calculated. The procedure has been validated to very low oxygen pressures (Bergersen and Turner, 1975).

Mitochondria (to a final cytochrome aa_3 content of 50 to 500 nmol l^{-1}) were suspended in solutions containing mannitol (225 nmol l^{-1}), sucrose (75 mmol l^{-1}), glucose (20 mmol l^{-1}), 10 mmol l^{-1} sodium Hepes buffer, pH 7.2, 10 mmol l^{-1} potassium phosphate buffer, pH 7.2, magnesium chloride (1.0 mmol l^{-1}), sodium glutamate (5 mmol l^{-1}), L-malic acid (2.5 nmol l^{-1}), hexokinase (25 units ml^{-1}), superoxide dismutase (1.5 units ml^{-1}), catalase (1.5 units ml^{-1}) and myoglobin or other hemoglobin to the desired concentration. The mitochondrial suspension was held in a modified Thunberg cuvette with a 1 cm light path and equilibrated with a wet gas stream at the desired initial P_{O_2} [1.3 kPa (10 mmHg) when myoglobin is used; 0.4 kPa (3 mmHg) when leghemoglobin or

other high-affinity hemoglobins are used]. Oxygen consumption is initiated by introducing ADP (final concentration, 500 μmol l^{-1}) from a side arm, and the absorbance at a single wavelength is followed at 25°C as a function of time (typically 2.5 to 5.0 min). Depending on the concentration of myoglobin, convenient wavelengths are 437, 581 or 625 nm.

ADP, approximately 50 μmol 'free' ADP kg^{-1} wet mass tissue in resting cardiac myocytes (Doeller and Wittenberg, 1991), is believed to play an important role in controlling mitochondrial respiratory rate *in situ*. The concentration used here, 500 μmol l^{-1}, is 10-fold greater than that in the myocyte, and the respiratory rate is expected to be independent of ADP concentration. Added glucose, Mg^{2+} and hexokinase together serve to regenerate ADP from ATP, maintaining the ADP concentration constant. Mg^{2+} is required for the action of hexokinase but also regulates mitochondrial-specific activity and, when present in excess, stimulates state IV mitochondrial respiration (Toth et al., 1990). The concentration used here, 1 mmol l^{-1}, is close to that found in heart, 0.51–0.85 mmol kg^{-1} wet mass tissue (Gupta et al., 1983; Murphy et al., 1989; Gupta and Wittenberg, 1991), and is not sufficient to stimulate state IV respiration significantly.

Calculation of the oxygen pressure at half-maximal rate of mitochondrial oxygen uptake

During the progress of the spectrophotometric assay, the slope of the traces, reflecting the rate of mitochondrial oxygen uptake, remains essentially constant as consumed oxygen is drawn predominantly from hemoglobin-bound oxygen and the rate of consumption remains independent of oxygen pressure. This slope is taken as maximal. The points of half-maximal slope were determined by numerical differentiation of relevant portions of the digital spectrophotometric records. The fractional saturation with oxygen of the hemoglobins at these points was calculated from the spectrum at the time of interest, the initial fully oxygenated spectrum and the final fully deoxygenated spectrum, using molar extinction coefficients of the difference in absorbance at a given wavelength between the oxy to deoxy proteins, determined for each protein. For myoglobin these are: 73.8, 5.93 and 1.2 mmol l^{-1} cm^{-1} at 437, 581 and 625 nm, respectively. Oxygen uptake is expressed as mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1}.

Determination of mitochondrial oxygen uptake using trace concentrations of myoglobin as a reporter

Trace amounts of myoglobin, ~5 μmol l^{-1}, too small to supply significant oxygen, were used as reporters of oxygen pressure in the range of lower P_{O_2} where the polarographic method is no longer applicable. Desaturation of added myoglobin is monitored spectrophotometrically. This procedure permits measurement of the rate of mitochondrial oxygen uptake below approximately 0.7 kPa (5 mmHg) where oxygen uptake is limited by solution P_{O_2}. Measurements are limited to P_{O_2} 0.25 to 0.4 kPa because only a limited concentration of oxygen is available at lower P_{O_2}.

Results

Validation of the spectrophotometric assay procedure

No detectable ferric protein is formed in the presence of superoxide dismutase and catalase. Control experiments gave identical results whether or not the cuvette contents were stirred; completely filled cuvettes with no gas phase were used in these control experiments. Accordingly, the contents of the Thunberg cuvette were not stirred in the experiments reported here. Material exchange between the gas phase and the aqueous solution in the lower part of the cuvette, where the light beam passes, is very slow. Control experiments indicate that it may be neglected in these brief experiments. The mitochondria settle slowly, so that the number entering and leaving the light path is the same, and mitochondrial density in the light path does not change during the course of these relatively brief experiments.

The conditions of the spectrophotometric assay of mitochondrial oxygen consumption differ radically from those of the familiar polarographic assay. Mitochondrial density is approximately 100-fold greater; ADP concentration ranges from 4- to 50-fold greater during the course of the determination; oxygen pressure is markedly less than that used in the polarographic assay. An experiment, presented in Fig. 1, was designed to show that the parameters of mitochondrial function are not much changed. The sharp breaks in the rate of oxygen uptake, where ADP is calculated to be exhausted, evidence tight coupling of oxygen uptake to ADP usage. Initial specific activities are ~200 mol O_2 (mol cytochrome

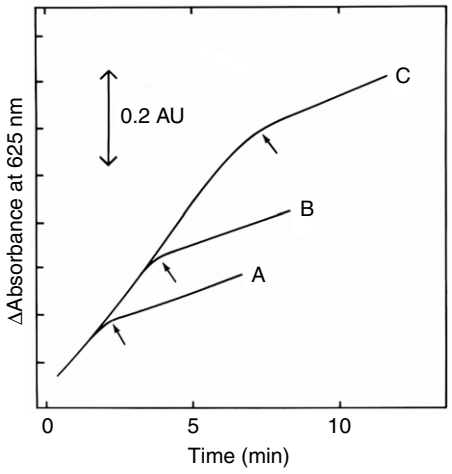

Fig. 1. Oxygen consumption reported by myoglobin deoxygenation monitored at 625 nm is determined in mitochondrial suspensions containing myoglobin and limiting amounts of ADP. ADP is exhausted at the arrows. Myoglobin, 500 μmol l^{-1}. A, 500 μmol l^{-1} ADP; B, 1000 μmol l^{-1} ADP; C, 2000 μmol l^{-1} ADP. Initial rates are ~200 mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1}. P/O ratios are: 2.3, 2.4 and 2.4, respectively, with respiratory control indices of 3.1–3.4. The P/O ratio of this preparation of mitochondria, determined polarographically, was 2.4 with RCI>6. This shows that mitochondrial oxygen uptake is tightly coupled to phosphorylation when the mitochondria are consuming myoglobin-bound oxygen. AU, absorbance units.

$aa_3)^{-1}$ min^{-1}. P/O ratios are: 2.3, 2.4 and 2.4 at initial ADP concentrations of 500, 1000 and 2000 μmol l^{-1}, respectively, with respiratory control indices of 3.1–3.4. These are comparable to those found in the polarographic assay: P/O ratio 2.4, with RCI>6 (*N*=24). This shows that mitochondrial oxygen uptake, measured in this assay, is tightly coupled to ADP usage and proceeds at rates commensurate with state III oxygen uptake measured polarographically.

Myoglobin does not interact with the mitochondrial surface

The polarographically measured rate of oxygen uptake by a suspension of pigeon heart mitochondria is changed only slightly by the addition of 500 μmol l^{-1} oxymyoglobin, a concentration greater than that in the pigeon ventricle, approximately 200 μmol kg^{-1} wet mass tissue (Schuder and Wittenberg, 1979), Fig. 2. The P/O ratio, likewise, was not affected by the presence of 500 μmol l^{-1} myoglobin. As discussed below, each of the six radically different hemoglobins used in these experiments supported mitochondrial oxygen uptake to approximately the same extent, Table 1 and Table 2.

Progress of hemoglobin deoxygenation during the experiment

These experiments explore low oxygen pressures comparable to sarcoplasmic oxygen pressure, where oxygen uptake would be limited by oxygen availability in the absence of hemoglobin or myoglobin. The progress of experiments in which deoxygenation of myoglobin (P_{50}=0.09 kPa; ~0.7 mmHg) or leghemoglobin *c* (P_{50}=0.009 kPa; ~0.07 mmHg) reports mitochondrial oxygen uptake is presented in Fig. 3. The ordinate reports hemoglobin oxygenation. Initially, oxygen is present in small excess; the hemoglobin is largely or fully oxygenated, and the traces curve downward as mitochondrial respiration draws on reserves of both dissolved and hemoglobin-bound oxygen. Subsequently, the store of dissolved oxygen becomes small relative to the store of hemoglobin-bound oxygen, equilibrium between oxyhemoglobin and free oxygen buffers the rate of change of oxygen pressure, and the traces approach linearity. The rate of hemoglobin deoxygenation now reports a near-steady-state rate of hemoglobin-supported mitochondrial oxygen consumption.

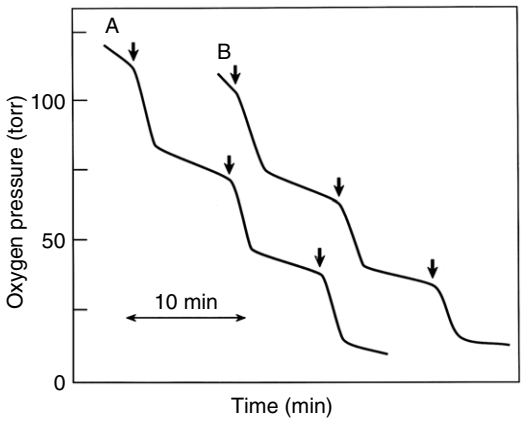

Fig. 2. Oxygen pressure in a suspension of mitochondria reported polarographically as a function of time (1 torr=0.133 kPa). ADP (500 nmol) is added at the arrows. A, myoglobin absent; B, 500 μmol l^{-1} oxymyoglobin. Average oxygen uptake in the presence of adequate ADP is 193 and 157 mol O_2 (mol cytochrome $aa_3)^{-1}$ min^{-1} in the absence and presence of myoglobin, respectively. P/O ratios are 2.21 and 2.20. The concentration of myoglobin used here exceeds the volume-average concentration in pigeon heart, 209 μmol l^{-1} (Schuder et al., 1979). These results show that myoglobin in the solution does not affect the respiratory parameters of isolated mitochondria.

The oxygen pressure at half-maximal rate of oxygen uptake is reported by the points at which the rate of change of hemoglobin deoxygenation is half that during the earlier quasi-linear portion of the progress curve, indicated by the arrows in Fig. 3. The two progress curves differ. Half-maximal respiration in the presence of the high-affinity leghemoglobin occurs when the protein is 57%, oxygenated; that in the presence of the lower affinity myoglobin occurs when the protein is 94%, deoxygenated. Accordingly, mitochondrial function is independent of the nature of the supporting hemoglobin and its fractional saturation with oxygen.

Oxygen pressure at half-maximal rate of mitochondrial oxygen uptake

The oxygen pressures obtained at half-maximal mitochondrial oxygen uptake, supported by six hemoglobins with very different kinetic and equilibrium constants in their

Table 1. *Oxygen affinity and equilibrium constants of monomeric heme protein used*

Heme protein	$P_{1/2}$ (kPa)	$P_{1/2}$ (mmHg)	Combination constant (mol l^{-1} s^{-1}×10^{-6})	Dissociation constant (s^{-1})
Busycon Mb[a]	0.11	0.81	48	71
Horse Mb[b]	0.056	0.43	14	11
Lucina Hb I[c]	0.044	0.34	100	61
Lucina Hb II[c]	0.021	0.16	0.39	0.11
Soybean Lb c_1[d]	0.003	0.021	124	4.9
Gasterophilus Hb[e]	0.009	0.067	10	1.2

Hb, hemoglobin; Mb, myoglobin; Lb, leghemoglobin. Kinetic constants from: [a]Busycon Mb (Schreiber and Parkhurst, 1984); [b]Horse Mb (Schenkman et al., 1997); [c]Lucina Hb I and Hb II (Kraus and Wittenberg, 1990); [d]Leghemoglobin *c* (Martin et al., 1990); [e]Gasterophilus Hb (Phelps et al., 1972).

Table 2. *Oxygen pressure and fractional saturation of heme proteins at half-maximal oxygen uptake of state III isolated cardiac mitochondria*

Heme protein	Fractional saturation (%)	Oxygen pressure (kPa)	Oxygen pressure (mmHg)
Busycon Mb	3.9	0.0047	0.035
Horse Mb	4.7	0.0094	0.071
Lucina Hb I	9.1	0.0024	0.018
Lucina Hb II	24	0.0067	0.050
Soybean Lb *c*	41	0.0067	0.050
Gasterophilus Hb	31	0.0015	0.011
Mean		0.0052	0.040

Hb, hemoglobin; Mb, myoglobin; Lb, leghemoglobin.

reactions with oxygen (Table 1), are presented in Table 2. It is apparent that nearly the same oxygen pressure (range 0.0015–0.0095 kPa; 0.011–0.071 mmHg. Average P_{O_2}= 0.005 kPa; 0.040 mmHg) prevails at half-maximal oxygen delivery by each of the hemoglobins. There is no correlation of half-maximal rate with oxygen affinity of the proteins nor with the combination or dissociation rate constants. The values presented in Table 2 are roughly comparable to the P_{O_2} for half-maximal respiration of mitochondria isolated from a variety of sources, the so-called K_m for oxygen (reviewed by Wilson et al., 1988). Even the highest value (≈0.01 kPa; ~0.10 mmHg), encountered in mitochondria where specific activity has been roughly doubled, falls within the envelope of values reported for K_m.

Mitochondrial oxygen uptake as a function of hemoglobin concentration

Mitochondrial oxygen uptake is enhanced in the presence of myoglobin. We examined the relation between myoglobin concentration and this enhancement. The rate of mitochondrial oxygen uptake, measured by the slope of the near-linear portion of the progress curve (Fig. 3), increases monotonically with hemoglobin concentration to attain a plateau where oxygen uptake is independent of hemoglobin concentration (Fig. 4). The functions presented in Fig. 4 are the same for leghemoglobin, myoglobin and Busycon myoglobin (data not shown), proteins that differ 10-fold in the kinetics and equilibrium of their reactions with oxygen (Table 1). Oxygen uptake is also independent of the degree of hemoglobin oxygen saturation within the linear range of experiments presented in Fig. 3. These findings confirm the conclusion that hemoglobin-dependent oxygen uptake does not involve reaction of the hemoglobin with the mitochondrial surface. The maximum rates of uptake of hemoglobin-delivered oxygen do not differ significantly from the rates of state III oxygen uptake determined polarographically in the absence of hemoglobins. We conclude that added oxyhemoglobins do not change mitochondrial-specific activity. Instead they relieve a limitation to mitochondrial oxygen uptake imposed by limited availability of dissolved oxygen at low P_{O_2}.

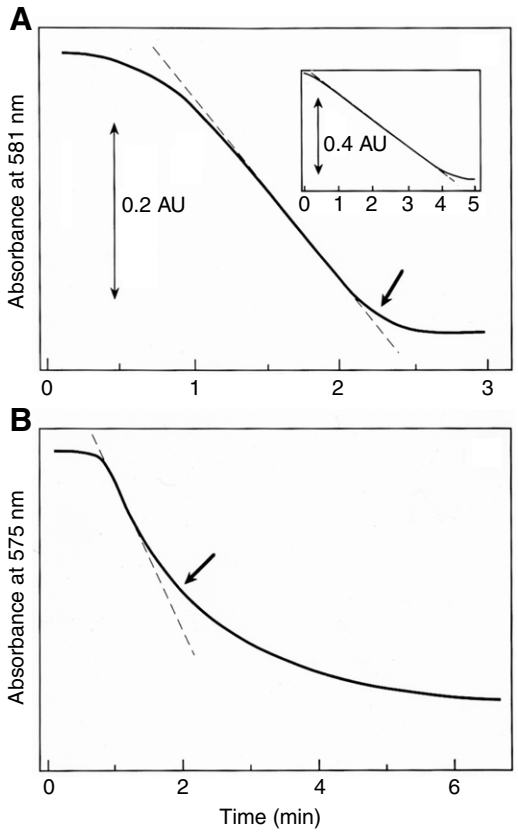

Fig. 3. Myoglobin and leghemoglobin deoxygenation as functions of time in hemoglobin-containing suspensions of mitochondria. Initially the hemoglobins are fully oxygenated. Deoxygenation is down. (A) Myoglobin, 50 μmol l^{-1}; mitochondria (as cytochrome aa_3) 170 nmol l^{-1}, 10 mm light path. (A, inset) Myoglobin 500 μmol l^{-1}; mitochondria (as cytochrome aa_3) 700 nmol l^{-1}, 2 mm light path. (B) Leghemoglobin *c*, 50 μmol l^{-1}; mitochondria (as cytochrome aa_3) 65 nmol l^{-1}, 10 mm light path. The broken lines are drawn at a tangent to the nearly linear portions of the curves. The arrows indicate the points at which the rates of mitochondrial oxygen uptakes are half that during the near-linear portion of the progress curves. The quasi-steady state oxygen uptakes calculated from the slopes of the broken lines are 204 and 358 mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1} for myoglobin and leghemoglobin, respectively. The oxygen pressure at half-maximal oxygen uptake is found to be the same for two proteins for which affinities differ 10-fold (see Table 2). This shows that the P_{O_2} for half-maximal mitochondrial oxygen uptake is not related to the oxygen affinity of the hemeprotein supplying oxygen. AU, absorbance units.

Effects of increased mitochondrial-specific activity

Mitochondria of heart and red skeletal muscle adapt their rate of oxidative phosphorylation to meet very large, say 10- or more-fold, changes in steady-state work output of the muscle. Accordingly, it is of interest to investigate the response of myoglobin-dependent oxygen delivery to change in mitochondrial-specific activity. Approximately doubling mitochondrial-specific activity roughly doubles the maximum rate of oxygen uptake at the plateau and more than doubles the oxygen pressure required to achieve half-maximal rate to a

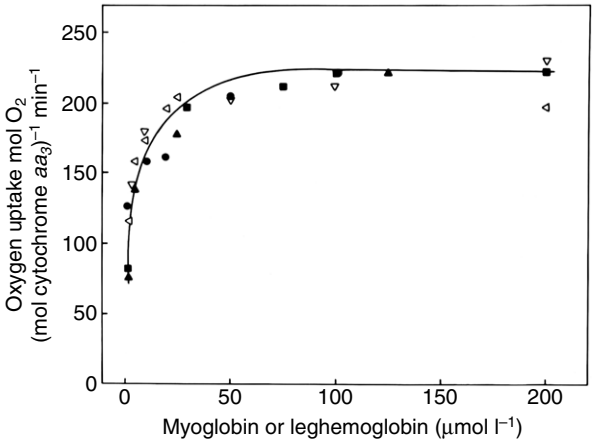

Fig. 4. Mitochondrial oxygen uptake as functions of myoglobin or leghemoglobin concentration. Closed symbols, myoglobin; open symbols, leghemoglobin *c*. Different symbols represent different experiments. With the exception of the two lowest points, oxygen uptake rates are those reported spectrophotometrically by the near-steady-state rates of deoxygenation of myoglobin or leghemoglobin, as in Fig. 3. Each point is the average of measurements on seven (myoglobin) or two (leghemoglobin) preparations of isolated mitochondria. The two lowest points were determined using trace concentrations of myoglobin or leghemoglobin as a reporter of oxygen pressure. Oxygen uptakes are normalized to a plateau value determined by the polarographically determined specific activity. The ratios of the rates of oxygen uptake at the plateau of this figure to those determined polarographically were 0.91, 0.77 and 1.30 in experiments using myoglobin and 0.76, 0.96 and 0.92 in experiments using leghemoglobin.

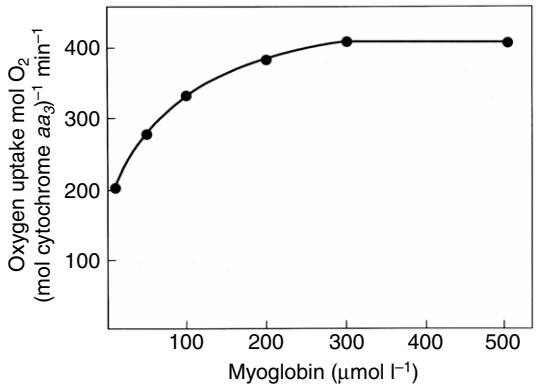

Fig. 5. Mitochondrial oxygen uptake as a function of myoglobin concentration using NADH (1 mmol l^{-1}) as the sole substrate. For this experiment mitochondria were prepared by a less stringent procedure, making the mitochondria able to use exogenous NADH. Polarographically determined specific oxygen uptakes of this mitochondrial preparation were 134 and 412 mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1} using glutamate/malate or NADH (1 mmol l^{-1}), respectively, as substrate. The latter may be compared to the rate exhibited by mitochondria fully uncoupled by exposure to carbonyl cyanide m-chlorophenylhydrazone (CCCP), 700 mol O_2 (mol cytochrome aa_3)$^{-1}$ min^{-1}.

value of ~0.01 kPa (~0.10 mmHg) (Fig. 5). The myoglobin concentration (approximately 300 μmol l^{-1}) required to sustain the full respiratory rate is approximately fivefold greater than that required at lesser rates, and is comparable to the volume-average myoglobin concentration in pigeon ventricle, 209 μmol kg^{-1} wet mass (Schuder et al., 1979).

Discussion

Krogh in 1919 (Krogh, 1919) and Wyman (Wyman, 1966) and Murray (Murray, 1971) 50 years later described the gradients of oxygen pressure driving oxygen from capillary to mitochondrion. Wyman notes that myoglobin-facilitated oxygen diffusion vanishes at full oxygen saturation of myoglobin where the gradient of myoglobin oxygen saturation becomes zero. Muscles, accordingly, function at sufficiently low oxygen pressure to partially desaturate the myoglobin with oxygen somewhere in the sarcoplasm. Wittenberg and Wittenberg formulated a model for diffusional oxygen transport in red muscle and heart (Wittenberg and Wittenberg, 2003), taking into account the large volume of experimental work done in the years intervening since Wyman and Murray's studies. In the interim, conditions at the high-oxygen-pressure boundary of the system, the sarcolemna, and those existing within the bulk of the sarcoplasm had been defined. However, the conditions at the low-pressure boundary, the mitochondrial–sarcoplasmic interface, have resisted experimental enquiry. These conditions cannot be predicted from the equations of facilitated diffusion and are required for analytic solution of those equations. Knowledge of the conditions at the mitochondria–sarcolemna interface is required to understand oxygen delivery to cytochrome oxidase. Here, we use a model system to define the minimum oxygen pressure required to assure the diffusive flow of oxygen from oxymyoglobin to mitochondrial cytochrome oxidase. Mitochondria, isolated from hearts of adult pigeons, are suspended in solutions of diverse heme proteins, and, in the region of measurement, derive their oxygen solely from protein-bound oxygen. In this condition, myoglobin-facilitated diffusion of oxygen dissipates the gradients of oxygen pressure that would otherwise occur in the unstirred layers surrounding each mitochondrion, and the oxygen pressure at the mitochondrial outer membrane is the same as that determined in the bulk solution.

First, we find no evidence that myoglobin interacts with the mitochondrial surface to affect oxidative phosphorylation. Mitochondrial oxygen uptake was not affected significantly by addition of myoglobin to 500 μmol l^{-1}, 2.5 times the concentrations found in tissue. Likewise, Cole et al. reported that myoglobin (to 180 μmol l^{-1}) does not affect oxidative phosphorylation by isolated rat heart mitochondria (Cole et al., 1982). The oxygen pressure required for half-maximal mitochondrial uptake (Table 2) and the concentrations of hemoglobin required to achieve maximal, near-steady-state rates (Fig. 4) were not affected by the nature of the supporting hemoglobin. In nature, the kinetics of reaction of each

myoglobin with oxygen are highly adapted to the requirements of function in the particular tissue in which they occur (Wittenberg, 2007). We learn, from the simplified model used here, that these adaptations serve mainly to achieve a sufficient oxygen pressure at the mitochondrial surface. We recall that, within the nitrogen-fixing plant root nodule, cytoplasmic leghemoglobin is separated from the bacteroids by the peribacteroid membrane; leghemoglobin cannot be detected in the peribacteroid space, and the possibility of interaction with the bacteroid surface does not arise (Wittenberg et al., 1996).

Added oxyhemoglobins, we discover, do not change mitochondrial-specific activity. Instead they relieve a limitation to mitochondrial oxygen uptake imposed by limited availability of dissolved oxygen. The flow of oxygen into the mitochondria is restored to the value in the absence of diffusion limitation, and the respiratory rate returns to the value determined polarographically.

Myoglobin concentration in the model system, or in muscle, must be sufficient to support respiration, the rate of which is set by the mitochondria. The experiments in Figs 4, 5 demonstrate this and display a plateau at saturating myoglobin/leghemoglobin concentration. The myoglobin concentration sufficient to support a mitochondrial respiratory rate approximating that which exists in the working heart (Fig. 5) is comparable to the volume-average myoglobin concentration in pigeon ventricle. The myoglobin content of muscles has long been known to roughly parallel their content of cytochrome oxidase (Lawrie, 1953; Millikan, 1939; Wittenberg, 1970), and this present finding raises the possibility that the myoglobin content of muscle is optimized to be just sufficient to deliver the needed oxygen to the mitochondrial surface.

The central finding of this study is that an oxygen pressure at the mitochondrial outer membrane of approximately 0.005 kPa (0.04 mmHg) is sufficient to support half-maximal state III respiration of isolated cardiac mitochondria (Table 2). At body temperature the pressure required will be approximately twofold greater than that reported here at 25°C. It is also increased to perhaps 0.013 kPa (0.1 mmHg) at increased rates of oxygen usage (Fig. 5). These values, estimated using isolated mitochondria, differ by no more than 0.01 kPa (~0.1 mmHg) from the volume-average sarcoplasmic oxygen pressure previously shown to be required for half-maximal respiration of resting intact isolated cardiac myocytes (Katz et al., 1984; Wittenberg and Wittenberg, 1985).

Myoglobin will not cross the mitochondrial outer membrane, and cytochrome oxidase, located in the inner mitochondrial membrane and cristae, must be supplied by dissolved oxygen diffusing from the sarcoplasm. Because the mitochondrial outer membrane offers negligible impedance to the flow of oxygen, only a very small oxygen pressure is required to sustain this flow. The oxygen pressure at the mitochondrial outer membrane actually far exceeds this value and, through the action of myoglobin-facilitated oxygen diffusion, reflects closely that in the bulk of the sarcoplasm. This, we reiterate, is controlled in working heart and muscle at a value near the P_{50} of myoglobin, 0.33 kPa (2.5 mmHg) at 37°C (Wittenberg and Wittenberg, 1989; Gayeski and Honig, 1991). We conclude that mitochondria of cardiac and red skeletal muscle myocytes do not lack for oxygen in normal steady states of sustained work.

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