Role of Myoglobin in the Oxygen Supply to Red Skeletal Muscle*

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The contribution of myoglobin to the oxygen uptake of red skeletal muscle was estimated from the difference in oxygen uptake with and without functional myoglobin. The oxygen uptake of bundles (25 mm long, 0.5 mm mean diameter) of muscle fibers teased from pigeon breast muscle was measured in families of steady states of oxygen pressure from 0 to 250 mm Hg. The oxygen-binding function of myoglobin, in situ in muscle fiber bundles, was abolished by treatment with nitrite or hydroxylamine, which convert oxymyoglobin in situ to high spin ferric myoglobin, or with phenylhydrazine, which converts oxymyoglobin to denatured products, or with 2-hydroxyethylhydrazine, which appears to remove myoglobin from the muscle. The oxygen uptake was again measured. At higher oxygen pressure, where oxygen availability does not limit the respiration of the fiber bundles, oxygen uptake is not affected by any of the four reagents, which is evidence that mitochondrial oxygen uptake is not impaired. At lower oxygen pressure, where oxygen uptake is one-half maximal, the steady state oxygen consumption is roughly halved by abolishing functional myoglobin. Under the steady state conditions studied, the storage function of myoglobin, being static, vanishes and the transport function stands revealed. We conclude from these experiments that myoglobin may transport a significant fraction of the oxygen consumed by muscle mitochondria.

Myoglobin unquestionably serves in some manner to aid the inflow of oxygen into cardiac and red skeletal muscle fibers, so that the demand for oxygen is met instantaneously (1, 2).

Millikan, in his classic study (3), showed that myoglobin in muscle undergoes rapid deoxygenation and oxygenation in response to fluctuations in oxygen supply and demand imposed by intermittent activity. He further showed that myoglobin maintains a steady level of partial oxygenation during sustained muscle contraction, implying that myoglobin in this circumstance functions in a very steep gradient of oxygen pressure from the capillary to the most remote mitochondrion (2).

Millikan foresaw that myoglobin might serve the body in three capacities: as an agent in oxygen transport, as an intracellular catalyst, or as an oxygen store. If, in fact, myoglobin is free to diffuse within the muscle cell, myoglobin-facilitated oxygen diffusion could assure the transport of oxygen (2, 4). Facilitated oxygen diffusion, that is, an oxygen flux brought about by translational diffusion of oxymyoglobin molecules, has been demonstrated in physical model systems (2). A priori we have no assurance that myoglobin serves this function in muscle tissue. In addition, leghemoglobin, the counterpart of myoglobin in plants, serves in the transfer of oxygen from the cytosol to a terminal oxidase (5). Finally myoglobin acts as a short period oxygen store to buffer fluctuations in the rate of flow of oxygen from the blood to the oxidizing enzymes of the mitochondria, thereby stabilizing the oxygen supply to the beating heart (1, 6-9).

Muscle oxygen demand, Millikan showed, rises instantaneously with the onset of contraction (3), and oxygen supply may be limited as the contracting muscle constricts the blood vessels. In this extreme circumstance myoglobin acts as an oxygen buffer, supplying oxygen to the mitochondria during contraction and taking on oxygen from the capillary blood during relaxation. On the other hand, in many muscles, fluctuations in supply and demand are strongly damped, and the muscle operates in a family of steady states in which oxygen supply and demand are in instantaneous balance (2). In this circumstance oxygen flows continuously to the mitochondria.

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from outside the muscle fiber, and myoglobin assumes a purely transport function. It is apparent that storage and transport are not necessarily separate functions but are extremes of a continuum, in which storage predominates during changing states of the muscle and transport is dominant in steady states.

Direct evidence that muscle oxygen supply depends on myoglobin is lacking. The present study was designed to address this problem and to answer the question: does myoglobin augment steady state muscle oxygen uptake? We find that it does. Oxygen uptake of muscle fiber bundles was measured in a series of steady states, and the results were expressed graphically as relations between steady state oxygen uptake and ambient oxygen pressure. The oxygen-binding function of myoglobin, in situ in the bundles of muscle fibers, was abolished by chemical treatment, and the contribution of myoglobin toward the oxygen uptake of the muscle fiber bundles was estimated from the difference in the steady state oxygen uptake with and without functional myoglobin.

**EXPERIMENTAL PROCEDURE**

**Optical Spectra—**Reflectance difference spectra of slabs of pigeon breast muscle were determined using a Cary model 17 recording spectrophotometer equipped with an integrating sphere (Cary cell space total diffuse reflectance accessory).

**Bundles of Muscle Fibers—**Adult pigeons were decapitated and bled from the neck vessels. Bundles of muscle fibers were teased from the breast muscle by blunt dissection following the natural divisions of the tissue, with care not to stretch the bundles or tear them along their length. A typical fiber bundle is 25 mm long and about 0.5 mm in diameter, and it weighs about 15 mg.

**Perfusion—**This was Krebs' improved Ringer's II solution (10) with 40 mM succinate as substrate. The perfusate was equilibrated with known gas mixtures containing 5% CO₂ and varying proportions of O₂ and N₂.

**Chamber for Measurement of Steady State Oxygen Consumption—**The chamber depicted in Fig. 1 was constructed from a 100-ml syringe. Vigorous stirring without undue tissue destruction is achieved by the magnetically driven rotating drum. The chamber volume is 40 ml, the flow rate is 1 ml per min, and the assembly is housed in a water bath maintained at 37 ± 0.05°. Oxygen pressure in the inlet and outlet streams was measured using a Radiometer (Copenhagen) E 5045/0 oxygen electrode and PHM-72 digital acid base analyzer equipped with a PHA 932 pO₂ module, and recorded continuously using a strip chart recorder.

**Measurement of Steady State Oxygen Consumption—**Tissue, 250 to 800 mg, is placed in the chamber. At the outset, the pO₂ measured at the outlet electrode declines with tissue oxygen uptake until oxygen consumption is balanced by oxygen entering in the input stream, and a steady state is achieved (half-time to achieve a steady state is about 5 min). The oxygen pressure of the input stream is then changed abruptly and the oxygen pressure at the new steady state is noted when it reaches a constant value. As many as 15 step changes in pO₂ may be made using the same sample of tissue. The time course of a typical experiment is presented in Fig. 2. Oxygen consumption is calculated from the difference in oxygen pressure readings of the two electrodes when the steady state is reached, the flow, and the solubility of oxygen in water at 37°. For this difference to be meaningful, the oxygen pressure of the output line must always be greater than zero.

**Measurement of Mitochondrial Oxygen Consumption—**Mitochondria were prepared from pigeon breast muscle by the procedure of Chance and Haghara (11), and the oxygen uptake of suspensions (0.2 mg of mitochondrial protein per ml) was measured at 37°.

**RESULTS**

**Reactions of Muscle Myoglobin—**These were revealed by reflectance difference spectra of reagent-treated minus untreated slabs of pigeon breast muscle (Fig. 3). These difference spectra show that nitrite and hydroxylamine convert muscle myoglobin to high spin ferric myoglobin. Although purified oxymyoglobin reacts with 2-hydroxyethylhydrazine to give predominantly the hydroxyethylhydrazine adduct of ferric myoglobin,† muscle fiber bundles incubated in the presence of 3 mM hydroxyethylhydrazine (37°) lose the color of myoglobin and become almost white. Reflectance difference spectra show only myoglobin loss from the muscle slab. Muscle fiber bundles exposed to phenylhydrazine assume the muddy green color of denatured myoglobin.

**Reversibility of Effect of Nitrite on Muscle Myoglobin—**Bundles of pigeon breast muscle fibers were examined using a microscope fitted with a microspectroscope ocular (Zeiss) or with a Hartridge reversion spectroscope (Beck, London) in place of the ocular. Ferrous (oxy- and deoxy-) myoglobin disappears in a population of fiber bundles incubated with 50 mM sodium nitrite (the broad bands of the product, ferric myoglobin, are not easily seen). When the fiber bundles are rinsed free of nitrite, ferrous myoglobin reappears with a time course of about 20 min at room temperature.

**Tissue Preparation and Muscle Fiber Bundle Thickness—**Pigeon breast muscle is rich in myoglobin, 0.20 to 0.25 mmol per kg wet weight of tissue. The majority of the fibers contain predominantly the hydroxyethylhydrazine adduct of ferric myoglobin,† which is reduced by 2-hydroxyethylhydrazine to give ferryl hemoglobin as the initial product (personal communication).

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†J. Peisach finds that oxyhemoglobin is reduced by 2-hydroxyethylhydrazine to give ferryl hemoglobin as the initial product.
myoglobin and are rich in mitochondria (12–15). They are homogeneous in size, are about 30 μm in diameter (14, 16), and could be classified as “red” fibers, with a mitochondrial distribution pattern (14) more typical of “intermediate” fibers (see Ref. 17 for a discussion of muscle fiber types). These red fibers form the core of each fiber bundle, with “white” fibers (about 22% of the total) arrayed at the periphery (14, 16).

The muscle is easily dissected into bundles of fibers. These are irregularly polygonal in cross section. Their shortest dimension in cross section, either when dissected free or in blocks of muscle, was measured with an ocular micrometer and found to range from 300 to 900 μm with a mean of 500 μm.

Degree of Oxygenation of Myoglobin in Muscle Fiber Bundles—Muscle fiber bundles surrounded by a flowing stream of 5% CO₂ in wet air were examined at 37° with a microscope fitted with a Zeiss microspectroscope ocular. The absorption bands of oxymyoglobin near 543 and 581 nm were faint, indicating that the myoglobin was substantially deoxygenated. These bands became very intense when tissue oxygen uptake was abolished by cyanide.

Limitation Imposed by Fiber Bundle Size—In an early study with Dr. P. P. J. M. Bindels,* we found that the rate of oxygen uptake depends on the rate of change of ambient pO₂, and is constant only at constant pO₂. For this reason we have made all measurements of oxygen uptake in steady states of constant pO₂.

Steady State Oxygen Uptake of Pigeon Breast Muscle—The results of four typical experiments using separated muscle fiber bundles are presented in Fig. 4. Oxygen uptake increases monotonically with pO₂, approaching an apparent plateau near 150 mm Hg, where oxygen uptake becomes nearly independent of oxygen pressure. Half-maximal rate occurs near 30 mm Hg, but substantial uptake is maintained to 5 mm Hg or less.

Caldwell and Wittenberg (18) report that relations similar to those of Fig. 4 obtain for slices of a variety of mammalian tissues.

The oxygen uptake of blocks of tissue, six layers of fiber bundles thick, followed a relation with oxygen pressure (Fig. 4) similar to that of separated fiber bundles, but the oxygen uptake was less.

The oxygen consumption of muscle fiber bundle preparations ranged from 30 to 60 μl of O₂ min⁻¹g⁻¹ wet weight. The relation between steady state oxygen uptake and oxygen pressure is independent of the order in which the measurements are made (Fig. 4), the rate of stirring, and the pH of the Krebs medium from pH 7.2 to pH 7.8. In the absence of exogenous substrate, oxygen uptake was low and variable. Addition of 40 mM succinate brought oxygen uptake, at high pO₂ where oxygen is not limiting, to a maximal value. The addition of glucose, 14 mM, separately or together with succinate, did not further increase the rate. Two uncouplers of the mitochondrial electron transport chain, 2,4-dinitrophenol (800 μM) and carbonyl cyanide m-chlorophenyl hydrazone (50 μM), were without effect.

Effect of Carbon Monoxide—It was not found possible to block the oxygen-binding function of myoglobin by reaction with carbon monoxide without simultaneously inhibiting cytochrome oxidase.

Lack of Effect of Symmetric Dicarbethoxyhydrazine and Ethyl-P-carboxymethylhydrazine—Neither of these reagents, which do not react with purified myoglobin, at respectively 0.5 mM and 15 mM concentration, changed the steady state oxygen consumption of muscle fragments.

Effect of Reagents Which Abolish Oxygenation of Myo-
The oxygen uptake of pigeon breast muscle fiber bundles at low pO2 is diminished in the presence of nitrite (Fig. 5), hydroxylamine (Fig. 6), or 2-hydroxyethylhydrazine (Fig. 7). Likewise the oxygen uptake of muscle fiber bundles pretreated with hydroxylamine (Fig. 8) or with phenylhydrazine (Fig. 9) is diminished at any pO2 where oxygen uptake is dependent on oxygen pressure. These effects vanished at high pO2 and the oxygen uptake becomes the same in the presence and absence of reagent treatment.

The effect of nitrite is reversible. Fig. 10 presents the results of an experiment in which nitrite-treated muscle fiber bundles were washed free of nitrite and subsequently incubated in Krebs-Ringer solution until the red color and absorption bands of oxymyoglobin (microspectroscope optical) were restored. The oxygen consumption was restored to the same level as that of a parallel preparation of untreated muscle fiber bundles.

The effect of hydroxylamine on steady state oxygen uptake was found negligible at 0.1 mM hydroxylamine, it was substantial at 0.25 mM, and it reached a plateau of maximal magnitude near 0.5 to 1.0 mM.

Effects of Reagents on Oxygen Uptake of Isolated Mitochondria—Nitrite (25 mM) inhibited the oxygen uptake of a mitochondrial preparation about 20%, at all pO2 from 5 to 110 mm Hg. This inhibition vanishes in the presence of 135 μM myoglobin, which becomes ferric and may serve to trap inhibitory products formed from nitrite. 2-Hydroxyethylhydrazine (5 mM) is without effect on mitochondrial oxygen uptake either in the presence or absence of ADP. Hydroxylamine (0.3 mM) inhibits mitochondrial oxygen uptake about 30 to 50%, independent of pO2 from 10 to 200 mm Hg. The effect of phenylhydrazine could not be tested because of nonenzymatic air oxidation of phenylhydrazine in neutral solution.

Effect of Cyanide—Cyanide is without effect on the reversible oxygenation of myoglobin. The concentration of cyanide used (195 μM) is about one-third that required to give 95% inhibition of cytochrome oxidase in a Keilin-Hartree preparation (19). Cyanide inhibits oxygen uptake of muscle fiber bundles at all oxygen pressures (Fig. 11).

Effect of Antimycin A—This inhibitor of mitochondrial electron transport does not affect the reversible oxygenation of myoglobin. Antimycin A inhibits the oxygen uptake of muscle fiber bundles at all oxygen pressures (Fig. 11). The concentration of antimycin A used in this experiment was 30 μg per ml but 0.6 μg per ml is sufficient.

**DISCUSSION**

Our intent in undertaking these experiments was to study the oxygen uptake of muscle under steady state conditions of oxygen supply and consumption where the storage function of myoglobin, by being static, vanishes, and the kinetic function of myoglobin in oxygen transport stands revealed. For this sufficient reason all rates of oxygen uptake were measured in steady states of constant ambient oxygen pressure. In addition, we report that steady state measurement is a requirement set by the long diffusion path for oxygen in the relatively large tissue fragments used. The apparent rate of oxygen uptake of the muscle fiber bundles depends strongly on the rate of change of oxygen pressure, and is constant only at unchanged ambient oxygen pressure. 8

Pigeon breast muscle was chosen because it is rich in myoglobin and because it can readily be dissected into bundles of fibers with minimum damage to the integrity of the tissue architecture. At oxygen pressures greater than 150 mm Hg, the oxygen uptake of pigeon breast muscle fiber bundles is independent of oxygen pressure (Figs. 4, 6, 7B, and 9). In this range, the oxygen uptake of these muscle fiber bundles is comparable to that of resting rat heart papillary muscles of about the same size (20), and is about one-third to one-half that of skeletal and cardiac muscles doing steady state work in situ with oxygen supplied from the capillaries (21).

Abolition of myoglobin function through the action of the reagents, which remove the oxygen-binding function of myoglobin, had no effect on muscle oxygen uptake in this range of oxygen pressure.

In the range of lower oxygen pressure, steady state oxygen uptake is dependent on oxygen pressure, rising steeply and monotonically from zero at zero oxygen pressure toward an apparent plateau at 150 mm Hg. The effect of myoglobin which we can demonstrate occurs in this range of oxygen pressure. This effect, the major finding of this study, is that abolition of the oxygen-binding function of myoglobin decreases the steady state oxygen uptake of muscle at any oxygen pressure within this range (Figs. 5 to 7, 9, and 10). Near the oxygen pressure for half-maximal oxygen uptake, treatment of the tissue with nitrite, hydroxylamine, or hydroxethylhydrazine (Figs. 5, 7A, 10) roughly halves the rate of oxygen uptake. To restore the original rate of oxygen uptake it is necessary to double the ambient oxygen pressure.

The simplest interpretation of these data is that myoglobin serves to transport oxygen to the mitochondria. In the range of lower oxygen pressure, this transport contributes a major fraction of the total oxygen uptake, the balance being the simple diffusive flow of dissolved oxygen.

We have used four reagents to abolish the oxygen-binding function of myoglobin. Although the mechanism of the initial attack of each of these reagents on oxyhemoglobin may be the same, the products differ. Two reagents, nitrite and hydroxylamine, convert oxyhemoglobin, both in situ in muscle (Fig. 3) and when isolated to high spin ferric myoglobin. One, hydroxethylhydrazine, converts isolated oxyhemoglobin to a mixture of products and attacks myoglobin in situ with apparent loss of the heme group so that at the end of an experiment the muscle fragments appear nearly white. The last reagent, phenylhydrazine, irreversibly denatures myoglobin, either purified or in situ, to a green product.

All of the myoglobin-reactive reagents have the potential to affect other heme proteins as well. The most convincing evidence that they exert their main effect on myoglobin function is the aforementioned fact that the effect of all the myoglobin-reactive reagents vanishes at high oxygen pressure, showing that the rate of mitochondrial oxygen uptake, per se, has not been affected. Further evidence for the integrity of mitochondrial function in reagent-treated muscle fiber bundles is the reversibility of the effect of nitrite (Fig. 10). When the tissue myoglobin returns to the ferrous, oxy- and deoxy-, state through the presumed action of tissue enzymes, the rate of oxygen uptake is restored to its original large value. Finally, we note that the pattern of inhibition obtained with the known inhibitors of mitochondrial oxygen uptake, cyanide and antimycin A (Fig. 11), is entirely different from that found with the myoglobin-reactive reagents. Cyanide and antimycin A inhibit the oxygen uptake of muscle fiber bundles at all oxygen pressures up to the highest examined.

Less direct evidence in support of the conclusion that 8 H. Adler, personal communication.
Fig. 5. Effect of nitrite on the steady state oxygen uptake of pigeon breast muscle fiber bundles. O, untreated tissue; ●, the same in the presence of 25 mM sodium nitrite.

Fig. 6. Effect of hydroxylamine on the steady state oxygen uptake of pigeon breast muscle fiber bundles. O and □, untreated fiber bundles; ●, the same in the presence of 0.25 mM hydroxylamine. Different symbols represent separate experiments.

Fig. 7. Effect of 2-hydroxyethylhydrazine on the steady state oxygen uptake of pigeon breast muscle fiber bundles; experiments in which 2-hydroxyethylhydrazine was injected into the chamber. Open symbols, untreated fiber bundles; Solid symbols, the same in the presence of 3 mM 2-hydroxyethylhydrazine. Different symbols represent separate experiments. A, an experiment chosen to demonstrate the usual magnitude of the effect. B, an experiment chosen to demonstrate the plateau of oxygen uptake at high oxygen pressure.

Fig. 8. Effect of 2-hydroxyethylhydrazine. Two experiments in which fiber bundles were pretreated with 2-hydroxyethylhydrazine until the color of myoglobin had disappeared. Open symbols, untreated fiber bundles; Solid symbols, fiber bundles which had been incubated at 37°C for 90 min in Krebs solution containing 100 mM 2-hydroxyethylhydrazine, rinsed for a total of 20 min at 37°C in four changes of reagent-free Krebs solution, and subsequently transferred to reagent-free Krebs solution in the measuring chamber. Different symbols represent separate experiments.

Fig. 9. Effect of phenylhydrazine on steady state oxygen uptake of pigeon breast muscle fiber bundles. Open symbols, untreated fiber bundles; Solid symbols, fiber bundles which were preincubated for 30 min at 4°C in a solution of phenylhydrazine, 5 mM, in Krebs solution, rinsed, and subsequently transferred to phenylhydrazine-free Krebs solution in the measuring chamber. Different symbols represent separate experiments.

Fig. 10. Reversibility of the effect of nitrite on the steady state oxygen uptake of pigeon breast muscle fiber bundles. ●, fiber bundles in the presence of 50 mM sodium nitrite. The sequence of measurements is from high to low PO2. O, the same fiber bundles after replacing the perfusing medium with nitrite-free Krebs solution and incubating for 1 hour at 37°C. The sequence of measurements is from low to high PO2.
condition for any carrier-mediated oxygen transport (2, 31, 32), and gradients of oxygenation have been demonstrated in working cardiac and skeletal muscle (3, 33-36). Thus myoglobin, in enhancing the oxygen flow, operates within steep gradients of oxygen pressure and partial oxygenation.

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Fig. 11. Effect of cyanide and of antimycin A on the steady state oxygen uptake of pigeon breast muscle fiber bundles. Open symbols, untreated fiber bundles. the same in the presence of 125 #M potassium cyanide. The sequence of measurements in the experiment with cyanide is indicated by the numbers of the data points. Fiber bundles in the presence of antimycin A, 30 #g per ml. Different symbols represent separate experiments.

FIG. 11. Effect of cyanide and of antimycin A on the steady state oxygen uptake of pigeon breast muscle fiber bundles. Open symbols, untreated fiber bundles. Fiber bundles in the presence of antimycin A, 30 #g per ml. Different symbols represent separate experiments.
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