Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes

(electron transport/heart cells/cytochrome oxidase)

BEATRICE A. WITTMENBERG* AND JONATHAN B. WITTMENBERG

Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Berta Scharrer, July 20, 1987 (received for review May 3, 1987)

ABSTRACT Myoglobin-mediated oxygen delivery to intracellular mitochondria is demonstrated in cardiac myocytes isolated from the hearts of mature rats. Myocytes are held at high ambient oxygen pressure, 40-340 torr (5-45 kPa); sarcoplasmic myoglobin is fully oxygenated. In this condition, oxygen availability does not limit respiratory rate; myoglobin-facilitated diffusion contributes no additional oxygen flux and, since oxygen consumption is measured in steady states, the storage function of myoglobin vanishes. Carbon monoxide, introduced stepwise, displaces oxygen from intracellular oxymyoglobin without altering the optical spectrum of the largely oxidized intracellular mitochondria. A large part, about one-third, of the steady-state oxygen uptake is abolished by carbon monoxide blockade of myoglobin oxygenation. The myoglobin-dependent component of the oxygen uptake decreases linearly with decreasing fraction of intracellular oxymyoglobin, with a slope near unity. Studies using inhibitors of mitochondrial electron transport indicate that myoglobin-delivered oxygen uptake depends on electron flow through the mitochondrial electron transport chain. We conclude that cardiac mitochondria accept two additive simultaneous flows of oxygen: a flow of dissolved oxygen to cytochrome oxidase and a flow of myoglobin-bound oxygen to a mitochondrial terminal. Myoglobin-mediated oxygen delivery supports ATP generation by heart cells at physiological ambient oxygen pressure.

Cytochrome oxidase, half-oxidized when ambient oxygen partial pressure (PO_2) is 0.07 torr (1 torr = 133 Pa) (16), in the circumstance described here experiences oxygen pressures 20- to 200-fold the pressure required to maintain the normal, largely oxidized, state seen in resting myocytes (16). Carbon monoxide in this circumstance blocks oxygenation of sarcoplasmic myoglobin selectively without perturbing the optical spectrum of intracellular cytochrome oxidase. We conclude that cardiac mitochondria accept two additive simultaneous flows of oxygen: the well-known flow of dissolved oxygen to cytochrome oxidase and a flow of myoglobin-bound oxygen to a mitochondrial terminal. The myoglobin-mediated oxygen flow supports ATP generation in the physiological range of oxygen pressure.

MATERIALS AND METHODS

Isolated Cardiac Myocytes. These were prepared from the hearts of mature rats by enzymatic digestion, purified on Percoll (Pharmacia) density gradients, and suspended to a final density of 0.5-1.0 x 10^6 cells per ml (19, 20).

Mitochondria. Mitochondria were prepared by the method of Palmer et al. (21) from adult rat hearts that had been perfused with balanced saline solution to remove erythrocytes.

Gas Partial Pressures and Oxygen Uptake. All measurements were made in steady states (7, 16) of constant oxygen pressure and oxygen uptake. The measuring chamber had both liquid and gas phases (16, 22). The gas phase composition was set by a mass flow controller (Tylan, Torrance, CA). Since carbon monoxide is not consumed, solution PCO is known from the composition of the gas phase. Solution oxygen pressure was monitored by a sensitive polarographic oxygen electrode and is the balance of oxygen entering and oxygen consumed by the myocytes. At constant temperature and stirring rate, oxygen uptake is known from the difference between solution PO_2 in the absence of myocytes (equal to gas-phase PO_2) and the actual solution PO_2 in the presence of myocytes, using a mass transfer coefficient that is determined daily (16, 22, 23). The temperature was 30°C in all experiments.

Optical Spectra. The chamber was held in a thermostatted block placed in the sample light beam of a Cary model 17 recording spectrophotometer (Varian) equipped with a Cary scattered transmission accessory. Data were acquired digitally from 650 to 350 nm, and difference spectra were constructed by subtraction, using an Aviv data acquisition system (Aviv Laboratories, Lakewood, NJ). The spectral contribution of myoglobin dominates the myocyte optical spectrum (16). Under the conditions of our experiments,

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; MbO_2, oxymyoglobin; MbCO, carbon monoxide myoglobin; PO_2, partial pressure of oxygen; PCO, partial pressure of carbon monoxide.

*To whom reprint requests should be addressed.
intracellular myoglobin is essentially completely ligated; oxygen and carbon monoxide myoglobin are the dominant species present. The contribution of myoglobin was calculated from the spectral changes occurring at 409 and 424 nm, when intracellular oxymyoglobin was converted fully to carbon monoxide myoglobin, and was subtracted from myocyte spectra to obtain a residue dominated by the spectral contribution of the mitochondria. The fraction of carbon monoxide myoglobin at any PCO was calculated from spectral changes observed at 409 and 424 nm. Excellent isosbesticity was maintained at 415 and 438 nm.

Energetics of Cardiac Myocytes. Suspensions of myocytes were incubated in the measuring chamber for 60 min. Oxygen uptake, intracellular ATP, intracellular phosphocreatine, and accumulated lactate (final minus initial) were determined as previously (16, 20).

RESULTS

The partition of intracellular myoglobin between carbon monoxide and oxygen, \( M = \frac{[\text{MbCO}]}{[\text{MbO}_2]} \times \text{PCO} / \text{P}_2 \), was determined graphically as the slope of the line in Fig. 1. The partition coefficient, \( M = 20 \) at 30°C, is not greatly different from that of purified rat heart myoglobin, \( M = 29 \) at 30°C, which we determined by conventional procedures (24, 25). The reasonable value of the slope, together with the linearity of the relation found, indicates near equilibrium between sarcoplasmic myoglobin, oxygen, and carbon monoxide.

The effect of carbon monoxide on steady-state respiration of cardiac myocytes is presented in Fig. 2. Within each experiment, the fraction of oxygen in the gas phase was held constant at 10-25% and the fraction of carbon monoxide was increased stepwise. Steady-state solution oxygen pressure, steady-state oxygen uptake, and optical spectra were monitored. The oxygen uptake of cardiac myocytes held at high PO2 decreases initially as PCO is increased, stays nearly constant in a prolonged plateau in which myoglobin is fully saturated with carbon monoxide, and finally, above 95% carbon monoxide, in the gas phase, declines to zero as carbon monoxide combines with cytochrome oxidase (Fig. 2a). The early decrease clearly follows the increasing fractional saturation of cytoplasmic myoglobin with carbon monoxide (Fig. 2b). This part of the respiration may be called the myoglobin-dependent oxygen uptake.

![Figure 1. Partition of intracellular myoglobin between oxygen and carbon monoxide. Data are from six experiments. Solution oxygen pressures ranged from 40 to 340 torr in different experiments. The partition coefficient \( M = 20 \) at 30°C.](image)

![Figure 2. Steady-state oxygen uptake of suspensions of cardiac myocytes as a function of carbon monoxide partial pressure. Respiratory rate (14.4 ± 3.3 nmol of oxygen per min per ml per 10^6 cells (mean ± SD; n = 29) in these experiments) is normalized, taking the uninhibited rate in each experiment as unity. Data are from three experiments. ■, 15% oxygen in the gas phase (solution PO2 = 80-90 torr); ●, 20% oxygen (solution PO2 = 100-120 torr); □, 25% oxygen (solution PO2 = 155-160 torr). (a) PCO = 0-730 torr. (b) The same data presented over a limited range; the fractional saturation of sarcoplasmic myoglobin in one experiment is presented (). The myoglobin-dependent oxygen uptake is taken as the difference between the uninhibited rate and the plateau value reached as sarcoplasmic myoglobin approaches saturation with carbon monoxide.](image)
50–300 torr) are in the state expected for myocytes in oxygen and are not affected by carbon monoxide.

Spectral features near 430, 537, and 563 nm, seen in the myocyte but not the mitochondrial spectra of Fig. 4, indicate that cytochrome $b$ of ubiquinone-cytochrome $c$ oxidoreductase [429 and 562–564 nm (refs. 26–28)], oxidized in fully oxidized isolated mitochondria, is detectably reduced in the aerobic isolated myocyte. Spectral contributions from cytochrome $b$ of succinate-ubiquinone oxidoreductase [424 and 560 nm (refs. 28–30)] or from cytochrome $b_5$ [423 and 556 nm (refs. 31–33)] are easily distinguished and are excluded.

The final decrease in respiration occurs at about 95% carbon monoxide in the gas phase (Po$_2$ = 700 torr; solution Po$_2$ = 7 torr). Concomitant with inhibition of respiration, optical spectra first indicate ligation of cytochrome $a_1$ to carbon monoxide. The spectral change is complete at 100% carbon monoxide in the gas phase (Fig. 5).

The spectral contribution of myoglobin cancels in the difference between spectra of cells exposed to 100% carbon monoxide and cells on the plateau region of Fig. 2 (Fig. 5, upper trace). The spectrum is essentially similar to the difference spectrum of isolated mitochondria exposed to carbon monoxide minus those in air (Fig. 5, lower trace), except for a feature near 563 nm in the mitochondrial spectrum ascribed to differential reduction of cytochrome $b$. Spectral features near 550, 445, and 605 nm seen in both traces indicate increased reduction of cytochrome $c$ and cytochrome oxidase in the absence of oxygen. A spectral feature near 593 nm seen in both traces is ascribed to carbon monoxide-ligated cytochrome $a_1$ of cytochrome oxidase. This confirms that intracellular mitochondria of cells exposed to carbon monoxide in air are in an oxidized steady state.

A small maximum near 419 nm appears in the difference between spectra of myocytes exposed to carbon monoxide and oxygen and those in oxygen alone (difference between

Fig. 3. Myoglobin-dependent oxygen uptake of suspensions of cardiac myocytes as a function of the fraction of sarcoplasmic myoglobin ligated to carbon monoxide. Myoglobin-dependent oxygen uptake, defined in the legend to Fig. 2, is normalized, taking the maximal value in each experiment as unity. Results of five experiments are shown. Two experiments with solution Po$_2$ = 40–60 torr; solution Po$_2$ = 70–90 torr; solution Po$_2$ = 100–120 torr; a single experiment with solution Po$_2$ = 340 torr (precision of measuring oxygen uptake is less in this experiment). Pco$_2$ at half saturation of sarcoplasmic myoglobin in different experiments was 3–15 torr. The solid line is a least-squares fit to the data of the four experiments represented by solid symbols. The slope is −1.1 (r = 0.96).

Fig. 4. Optical spectra of a suspension of cardiac myocytes, compared to spectra of isolated mitochondria. Bottom trace, spectrum of an oxygenated suspension of rat heart mitochondria. Middle trace, spectrum of a suspension of cardiac myocytes (10$^6$ cells per ml; solution Po$_2$ = 35 torr) minus the spectral contribution of 5.2 µM MbCO. At this solution oxygen pressure the intracellullar myoglobin is 96% oxygenated (16). Upper trace, spectrum of the same cell suspension (solution Po$_2$ = 36 torr; Pco$_2$ = 295 torr) minus the spectral contribution of 5.2 µM MbCO. This trace corresponds to a point on the respiratory plateau of Fig. 2, where the myoglobin-dependent respiration is fully inhibited, and intracellular myoglobin is fully saturated with carbon monoxide. The myocyte spectra are nearly the same in the absence and presence of carbon monoxide, differing only slightly in the Soret region.

upper and middle traces of Fig. 4, not presented). This is the only spectral change (other than the changes ascribed to

Fig. 5. Optical difference spectrum of cardiac myocytes. Intracellular myoglobin is fully saturated with carbon monoxide in each parent myocyte sample, and the spectral contribution of MbCO is cancelled in the difference. This is compared to a mitochondrial difference spectrum. Upper trace, spectrum of a suspension of cardiac myocytes (0.5 × 10$^6$ cells per ml) in carbon monoxide alone (Pco$_2$ = 730 torr) minus the spectrum of the same suspension, taken earlier (solution Po$_2$ = 195 torr; Pco$_2$ = 370 torr; this corresponds to about the midpoint of the respiratory plateau of Fig. 2). Lower trace, spectrum of a suspension of isolated rat heart mitochondria in the presence of succinate and carbon monoxide minus the spectrum of the same suspension taken earlier in the presence of oxygen. The traces are largely the same except for a greater prominence of a feature near 563 nm in the mitochondrial trace. Cytochrome $b$, to which this feature is ascribed, is always detectably reduced in myocytes (Fig. 4) and is fully reduced in anaerobic mitochondria.
myoglobin) consistently accompanying binding of intracellular myoglobin to carbon monoxide and is observed even when myoglobin-dependent oxygen uptake is decreased in the presence of antimycin, myxothiazol, or cyanide. This feature is not observed in spectra of isolated mitochondria exposed to 50% carbon monoxide in oxygen. Candidates for the origin of this difference are many. It may reflect increased reduction of cytochrome c or of cytochrome c1 of ubiquinone-cytochrome c oxidoreductase [reduced minus oxidized difference spectrum, 418, 523, and 553 nm (refs. 34 and 35)]. Or it may reflect a ferryl or a low-spin ferric form of myoglobin or possibly a minor perturbation in the steady state of cytochrome oxidase.

Inhibitors of the mitochondrial respiratory chain (36) were used to elucidate the relation of myoglobin-mediated oxygen uptake to mitochondrial function. Cyanide (1 mM) or myxothiazole (2 μM) inhibit 80–85% of the respiratory oxygen uptake of cardiac myocytes. In the presence of either, myoglobin-mediated oxygen flux is abolished. Oxygen uptake can be inhibited partially (50–60% of control) at lower concentrations of cyanide (10–50 μM) or of antimycin (0.6 μM). At this level of inhibition, difference spectra (30 μM cyanide minus control) exhibit features near 550 and 600 nm, attributed to increased reduction of cytochrome c and to perturbation of cytochrome oxidase. Difference spectra (antimycin minus control) exhibit well-resolved maxima near 430 and 563 nm, attributed to additional reduction of cytochrome b of ubiquinone-cytochrome c oxidoreductase (36, 37). In these partially inhibited states of cellular respiration, carbon monoxide (10–40% in the gas phase) brings about only a small inhibition (11 ± 5%; n = 7) of oxygen uptake in each experiment. Optical spectra indicate no further reduction of cytochrome c or perturbation of cytochrome oxidase. An increased absorbance is noted near 419 nm.

To demonstrate the effect of myoglobin blockade on cell energetics, intracellular mitochondria of cardiac myocytes were partially uncoupled by the proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), approximately doubling oxygen uptake at lower cellular ATP and phosphocreatine levels (Fig. 6). In the presence of CCCP, at both physiological and higher oxygen pressures, carbon monoxide blockade of myoglobin function does not change ATP concentration significantly, but it markedly decreases phosphocreatine concentrations (P ≤ 0.01) and markedly increases the rate of lactate accumulation (P ≤ 0.03). This indicates that blockade of myoglobin function results in a decreased rate of ATP generation by mitochondrial oxidative phosphorylation.

Increased oxygen uptake with CCCP indicates that oxygen diffusion to mitochondria is not rate limiting at the oxygen pressure used in these experiments.

**DISCUSSION**

We use carbon monoxide to block the oxygen-binding function of myoglobin in situ in the cardiac myocyte. The equilibrium binding of cytoplasmic myoglobin to oxygen (16) and the equilibrium partition of cytoplasmic myoglobin between oxygen and carbon monoxide (Fig. 1) are similar to those of purified myoglobin, indicating that ligand binding is not strongly modified in the intracellular environment. This justifies the use of carbon monoxide here. We note that attempts to use agents such as hydroperoxides to block intracellular myoglobin function in cardiac myocytes are thwarted by rapid reduction of intracellular ferric or ferryl myoglobin (7, 16, 38).

In many of the experiments presented here (Figs. 2 and 3) very high oxygen pressures (40–340 torr) were maintained in the medium in which the cells were suspended. Sarcoplasmic myoglobin was essentially fully oxygenated. Since the oxygen pressure difference between the extracellular medium and the intracellular mitochondria of isolated cells does not exceed 2–3 torr (15, 16), the mitochondria experienced large oxygen pressures. Optical spectra (Figs. 4 and 5) establish that cytochromes of intracellular mitochondria of myocytes exposed to carbon monoxide (≤50%) in the presence of much oxygen, conditions of the respiratory plateau of Fig. 2, are in a largely oxidized state unaltered by carbon monoxide. A large part, about one-third, of the total oxygen uptake of cardiac myocytes is abolished by carbon monoxide blockade of intracellular myoglobin function (Figs. 2 and 6). The myoglobin-dependent component of the cellular oxygen uptake decreases linearly with increasing fractional saturation of sarcoplasmic myoglobin with carbon monoxide (that is, decreasing fraction of oxymyoglobin), with a slope near unity (Fig. 3). Half inhibition was achieved at different Pco (3–15 torr) in experiments at different oxygen pressures, but always when monoxide was half saturated with carbon monoxide. Therefore, the effect of carbon monoxide is exerted on myoglobin alone and cannot be ascribed to inhibition of other cellular functions. We conclude that the myoglobin-dependent component of the oxygen uptake is proportional to the fraction of sarcoplasmic myoglobin combined with oxygen.

Myoglobin-dependent oxygen uptake disappears when oxidative phosphorylation is blocked by myxothiazol (2 μM) or cyanide (1 mM). It becomes small when the rate of electron throughput in the mitochondrial respiratory chain is diminished by antimycin (0.6 μM) or cyanide (10–50 μM). This suggests that myoglobin-mediated oxygen delivery requires electron flow through the cytochrome bc1 complex (complex III) and/or cytochrome oxidase (complex IV) of the respiratory chain.

**FIG. 6. Effect of carbon monoxide and CCCP on energetics of cardiac myocytes.** CCCP (when present) was 1.0 μM. Carbon monoxide pressure (when present) was 75 torr. Oxygen pressure was 1–5 torr in 15 experiments, 15 torr in 1 experiment, and 50 torr in 1 experiment; the results did not differ significantly. Although sarcoplasmic myoglobin is partially deoxygenated at solution P02 = 1–5 torr, there is no anoxic stimulus to lactate production until solution P02 is decreased to about 0.1 torr (16). Oxygen uptake and lactate accumulation are rates. ATP and phosphocreatine are steady-state concentrations. Results are expressed as fractions of the control values. These are as follows: oxygen uptake, 2.8 ± 0.6 nmol per min per mg of protein (n = 16); ATP, 29 ± 5 nmol per mg of rectangular cell protein of mitochondria containing intact cells (ref. 20) (n = 16); phosphocreatine, 40 ± 6 nmol per mg of rectangular cell protein (n = 15); lactate, 2.1 ± 0.4 nmol per min per mg of rectangular cell protein (n = 4).
Resting myocytes at physiological ambient PO2 (1–5 torr) maintain their energy reserves in the face of blockade of myoglobin function. Partial uncoupling of mitochondrial oxidative phosphorylation roughly doubles respiratory oxygen uptake and establishes a new steady state, simulating that in the working heart. The phosphocreatine reserve is less and ATP concentration is near a minimum working level. Carbon monoxide blockade of myoglobin oxygenation impairs the ability of these myocytes to meet the demand for ATP (Fig. 6). The steady-state concentration of intracellular ATP is itself conserved, but a sharp fall in the standing concentration of phosphocreatine and a dramatic increase in the rate of lactate accumulation indicate that the rate of oxidative phosphorylation has decreased, and the short fall in ATP generation has been compensated by a shift in the balance between phosphocreatine and creatine and by increased aerobic glycolysis to meet the fixed demand for ATP. Intracellular myoglobin function, therefore, supports ATP generation at extracellular oxygen pressures close to the pressure expected for intact cardiac muscle.

Oxymyoglobin supporting mitochondrial ATP generation may deliver its oxygen to mitochondria. Alternatively, sarcoplasmic oxymyoglobin might accept electrons from mitochondria, with concomitant reduction of heme iron-ligated oxygen to water. Our evidence cannot distinguish between these two formal possibilities. Either mechanism requires interaction between oxymyoglobin and mitochondria.

Myoglobin-dependent oxygen delivery to mitochondria, demonstrated here in myocytes, finds a strong parallel in leghemoglobin-mediated oxygen delivery to nitrogen-fixing symbiotic bacteria of the soybean root nodule. Oxygen delivered by leghemoglobin supports ATP generation in the intact nodule (39) and in a broken cell system (40–43). Dissolved oxygen, in contrast, although consumed rapidly, supports only limited ATP generation. The bacterial oxidase(s), presumably located in the cell membrane, is separated from leghemoglobin by the bacterial cell wall. We do not yet know how leghemoglobin-delivered oxygen is transferred across the bacterial wall, just as we do not understand how myoglobin-bound oxygen is delivered across the outer mitochondrial membrane to the inner membrane.

We conclude that a dominant function of myoglobin in the cardiac myocyte is myoglobin-mediated oxygen delivery to mitochondria. The myoglobin-mediated oxygen flow supports ATP generation at physiological oxygen pressures. We suggest that myoglobin-mediated oxygen delivery may contribute importantly to the ability of the heart to sustain maximum work output. Loss of this function may explain the cardiac toxicity of subacute carbon monoxide poisoning.

We thank Drs. C. A. Appleby, D. Mauzerall, and M. Wikstrom for helpful discussion and advice. Financial support for technical assistance. ATP work was supported in part by a grant in aid from the New York Heart Association (to B.A.W.), by Grants HL 19299 and HL 33655 from the U.S. Public Health Service (to B.A.W.), and by Research Grants DMB 84-16001 and PCM 84-16016 (to J.B.W.) from the U.S. National Science Foundation. J.B.W. is Research Career Program Awardee 1-K6-733 of the U.S. National Heart, Lung, and Blood Institute.