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Myoglobin and Mitochondria: Oxymyoglobin Interacts with Mitochondrial Membrane during Deoxygenation

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Abstract—The rates of oxygen uptake by rat liver mitochondria (MC) (native coupled, freshly frozen, and uncoupled by FCCP) have been measured polarographically in the absence (V_0) or presence (V_1) of 0.11-0.25 mM sperm whale MbO_2. Under the same standard conditions, the rate of sperm whale MbO_2 deoxygenation (V_2) has been studied spectrophotometrically in the presence of respiring MC. For freshly frozen MC, the dependence of V_1 and V_2 on the overall charge of MbO_2 has been investigated at pH 5.6-7.6, and the influence of other differently charged proteins (apomyoglobin, egg lysozyme, lactalbumin, and BSA) has been studied at pH 7.4. It is shown that the rate of mitochondrial respiration in the presence of MbO_2 increases by 10-30% ($V_1 > V_0$). No myoglobin effect is observed for FCCP-uncoupled MC (V_{max} does not change). The rate of MbO_2 deoxygenation is equal to the rate of oxygen uptake by mitochondria ($V_2/V_1 \sim 1$ at pH 7.2-7.5). At varying pH < 7.2, the V_2 values become markedly higher than V_1, evidently due to the increased MbO_2 positive charge and its stronger interaction with negatively charged mitochondrial membrane. At pH 7.4, on the contrary, V_2 is twice lower than V_1 in the case of negatively charged CM-MbO_2 (p*I* 5.2), which has carboxymethylated histidines. Positively charged lysozyme (p*I* 11) strongly inhibits MbO_2 deoxygenation (V_2) without affecting oxygen uptake by MC (V_0 and V_1). At the same time, apomyoglobin (p*I* 8.5), which is structurally very similar to the holoprotein, and both negatively charged lactalbumin (p*I* 4.4) and BSA (p*I* 4.7) have no substantial influence on V_2 and V_1. The MC membrane evidently has no specific sites for the interaction with myoglobin. Rather, the protein contacts with phospholipids of the outer membrane during MbO_2 deoxygenation, and electrostatic interactions are of great importance for this process.

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The most important condition for the functioning of animals is continuous delivery of oxygen to their tissues. Oxygen delivery for metabolic processes in cells is mediated by respiratory proteins: hemoglobin (Hb) in blood and myoglobin (Mb) in muscle. The rate of Mb oxygenation depending on O_2 concentration in solution is described by a hyperbolic curve (in contrast to sigmoid dependence for Hb) and exceeds 6-fold the rate of Hb oxygenation, which determines the physiological role of Mb [1]. When the partial O_2 pressure (P_{O_2}) in venous blood is 40 mm Hg, Hb and Mb are oxygenized by 60 and 95%, respectively.

The high affinity of Mb to oxygen and its high concentration in the muscles of animals, particularly those making prolonged dives in search of food, serve as the basis for the assumption that myoglobin functions as an "oxygen depot". The "depot" works in compliance with the curve of protein oxygenation in solution, i.e. O_2 is detached from oxymyoglobin (MbO_2) when P_{O_2} in a cell drops below a certain critical level. Hill [2] and Millikan [3] in the 1940s calculated that Mb of cardiac muscle can retain the O_2 amount sufficient for a complete cycle of contractions. By analogy, skeletal muscles can reserve an amount of O_2 sufficient for an 8-sec contraction under its maximal uptake [1].

Diffusion processes are very important for the systems of O_2 transfer and uptake. Hence, great attention has been given to experimental and theoretical studies of

Abbreviations: apo-Mb, apomyoglobin; CM-Mb, met-Mb carboxymethylated at histidine residues by bromoacetate; D_{Mb}, Mb diffusion coefficient; Hb, hemoglobin; MC, mitochondria; Mb, myoglobin; MbO_2, oxymyoglobin; met-Mb, metmyoglobin; P_{O_2}, partial oxygen pressure; P_{50}, Mb affinity to oxygen; S_{MbO_2}, degree of Mb saturation with oxygen.

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the phenomenon of O_2 diffusion facilitated by respiratory proteins, HbO_2 or MbO_2 [1]. Thus, the rate of transfer of labeled $^{18}O_2$ across porous membranes filled with MbO_2 solution accelerates 15-fold compared with metmyoglobin (met-Mb) solution [4]. However, the role of O_2 diffusion facilitated by myoglobin *in vivo* is unclear, because it is obvious that the protein cannot effectively compete with the diffusion of free oxygen. Nevertheless, it is believed that the intracellular O_2 transfer from sarcolemma to mitochondria rather than its storage is the main function of myoglobin in muscles [5-9]. The key points for the theory of this phenomenon are sufficiently high coefficients of lateral diffusion of Mb and its concentration in cells [10, 11].

It should be noted that Mb functioning in accordance with the "oxygen depot" and "facilitated diffusion" mechanisms is effective only at low P_{O_2} in cells (about 2.5-5 mm Hg) and high MbO_2 concentration gradients depending on the degree of saturation of the protein with oxygen (S_{MbO_2}). Modern experimental methods allow sufficiently exact estimation of these parameters. It has been shown that P_{O_2} values in working cardiac muscle are 11.4-25 mm Hg by different estimates (not 0.5-3.5 mm Hg as considered previously). At the same time, S_{MbO_2} in a heart working under heavy load does not fall below 85% and constitutes up to 92% of the total protein pool (not 10-30% as determined previously); i.e. neither extremely low P_{O_2} values nor high MbO_2 concentration gradients are registered inside the cell [12-14]. Diffusion coefficient of Mb (D_{Mb}) in intact skeletal muscles also proved to be 5-10 times lower than the values used previously to calculate the contribution of "facilitated diffusion" to oxygen delivery to cells, so that this contribution is negligible at $P_{O_2} > 13$ mm Hg and constitutes only 1.5-4% at lower P_{O_2} values [12, 15]. Calculations with the D_{Mb} value found recently for mouse heart [16], which is approximately 3-fold higher than D_{Mb} of skeletal muscles, nevertheless show that the contributions of free oxygen and myoglobin-bound O_2 are the same even at P_{O_2} = 1.8 mm Hg and MbO_2 concentration of 0.2 mM.

Thus, the effective functioning of Mb by the "oxygen depot" and "facilitated diffusion" mechanisms is very questionable. Neither of the mechanisms formulated in the framework of homogenous thermodynamics and kinetics assumes any interaction of the protein with cell structures or metabolites so that the Mb affinity to O_2 is taken constant in all model calculations. However, understanding of the actual mechanism of Mb functioning in a cell and its role in O_2 delivery to MC is not only important for solution of the fundamental problem of molecular biology and biochemistry, but also quite relevant in the applied aspect. One has to create the best conditions for supply with O_2 when constructing devices for cell culture and, accordingly, during mathematical modeling of these processes, to correctly assess Mb contribution and its need for introduction into the genes and cell culture for Mb expression [17].

We showed earlier [18] that the O_2 release from MbO_2 at physiological P_{O_2} values is possible only upon direct contact of the protein with MC. In the case when respiring MC are separated from an MbO_2 solution by a semipermeable membrane, no MbO_2 deoxygenation is observed even at near-zero O_2 concentration. Thus, the process of MbO_2 deoxygenation in the cell must include active interaction of the protein with the mitochondrial membrane, resulting in lower Mb affinity to the ligand.

The goal of the present work was to study the character of the Mb–MC interaction. Since the outer mitochondrial membrane, 50% of which is occupied with proteins, is negatively charged, the effect of the total charge of sperm whale myoglobin (p*I* 8.3) and chemically modified CM-MbO_2 (p*I* 5.2) carboxymethylated at histidines on spectrophotometrically measured MbO_2 deoxygenation rate (V_2) have been studied at pH 5.6-7.6. The role of electrostatic interactions in the system was assessed also by measuring MbO_2 deoxygenation rate (V_2) in the presence of other proteins: negatively charged monomeric lactalbumin (p*I* 4.4) and tetrameric bovine serum albumin (p*I* 4.7) and positively charged egg lysozyme (p*I* 11). The MC membrane was tested for the presence of myoglobin-specific proteins, channels, or phospholipid regions by investigating the effect of apomyoglobin, structurally homologous to the holoprotein but not binding O_2, on V_2. Also, the rates of O_2 uptake from solution by rat liver MC (native, FCCP-uncoupled, and freshly frozen) were measured by polarography under different conditions: without oxymyoglobin (V_0) and in the presence of 0.11-0.25 mM of a solution of sperm whale MbO_2 and various other proteins (V_1).

MATERIALS AND METHODS

Materials. Rat liver MC were isolated by a standard method using differential centrifugation in medium containing mannitol, 220 mM; sucrose, 70 mM; Hepes, 5 mM; and EGTA, 1 mM, pH 7.4. The final centrifugation was carried out at 3000*g* for 15 min (only large intact MC precipitated). Freshly isolated native coupled MC with the minimum rate of succinate respiration (V_{min}), fast-frozen at –18°C and freshly defrosted MC, and native MC uncoupled by FCCP (0.5 μM) with the maximum rate of succinate respiration (V_{max}) were used at a ratio of V_{max}/V_{min} = 4.5-6.5.

Metmyoglobin (met-Mb) from sperm whale skeletal muscles (17.8 kDa, fraction IV) was obtained and purified as described [19]. Oxymyoglobin was obtained under aerobic conditions by reduction of met-Mb with sodium dithionite, which was then separated by gel filtration on a Sephadex G-25 column.

Sperm whale met-Mb carboxymethylated at histidine residues (CM-Mb) was obtained with 0.2 M sodium bromoacetate; the reaction was performed in 0.1 M phos-

phate buffer, pH 7.0, at room temperature [20]. In 6-7 days, excess reagent was removed by intensive dialysis against 0.01 M phosphate buffer, pH 6.0, or gel filtration on a Sephadex G-25 column. Then the reaction mixture was fractionated by ion-exchange chromatography on CM-Sephadex C-25.

Apomyoglobin (apo-Mb) (16.2 kDa) was prepared from met-Mb by precipitation in acid acetone at –20°C [21]. After centrifugation and washing of the precipitate with cold acetone, the apoprotein was immediately dissolved in cold bidistilled water, dialyzed against 0.1 mM EDTA and water, and lyophilized. The resulting apo-Mb preparation contained no met-Mb (by absorption in the Soret band), and the content of α-helix was 59-60%.

Human α-lactalbumin (14.02 kDa) was isolated from milk by a method described previously [22] and kindly provided by V. E. Bychkova (Institute of Protein Research, Russian Academy of Sciences). The purity of the preparation determined by gel electrophoresis was no less than 98%.

Hen egg lysozyme (14.2 kDa) (Serva, Germany) and BSA (69 kDa) (Sigma, USA) were used without additional purification.

Chemical reagents: d-mannitol (analytical grade), sucrose (extra-pure grade), and Tris (Serva); Hepes (Gerbu, Germany); EGTA, KCl (extra-pure grade), and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) (Sigma) were used without additional purification. KH_2PO_4 and K_2HPO_4 for buffer solutions were recrystallized from water–alcohol mixture.

Absorption spectra. In the UV and visible regions, the absorption spectra were recorded using a Specord UV VIS spectrophotometer (Germany). Met-Mb concentration was determined by the molar coefficients of absorption ($mM^{-1}\cdot cm^{-1}$) $\varepsilon_{409} = 158$ and $\varepsilon_{505} = 9.5$; MbO_2 concentration was determined by $\varepsilon_{581} = 14.2$ [19, 20]. Apo-Mb, lactalbumin, lysozyme, and BSA concentrations were determined by $\varepsilon_{280} = 16$, 25.8, 38.5, and 26.4 $mM^{-1}\cdot cm^{-1}$, respectively [21, 22].

Kinetic measurements. The release of O_2 from MbO_2 in suspension of MC (mitochondrial protein concentration, 0.7-1 mg/ml) was registered at room temperature in the band of 581 nm, where the difference in absorbance of oxy- and deoxy-Mb was maximal ($\Delta\varepsilon_{581} = 6.4$ $mM^{-1}\cdot cm^{-1}$). Measurements were performed in the spectrophotometer chamber for turbid samples in cuvettes with optical pathlength of 0.2-0.5 cm to reduce the effect of light scattering. MbO_2 concentration in the samples was 0.11-0.25 mM; the incubation medium contained succinate (15 mM), EGTA (0.5 mM), KH_2PO_4 (5 mM), Hepes (10 mM), sucrose (150 mM), and KCl (100 mM, high ionic strength) or sucrose (250 mM) without KCl (low ionic strength). The pH values at high and low ionic strength were varied from 5.6 to 7.6.

Polarographic measurement of rate of O_2 uptake by MC. The measurement was performed under standard conditions using an Expert-001 thermooxymeter (Econix-Expert, Russia) equipped with a dissolved oxygen amperometric sensor, DKTP-O_2, in a 1-ml cell. The kinetic curve was recorded automatically using the manufacturer's software.

RESULTS

Oxygen uptake by MC and oxymyoglobin deoxygenation. The rate of O_2 uptake by native and freshly frozen rat liver MC increases in the presence of MbO_2 solution, 0.11-0.25 mM. The uncoupling effect of MbO_2 on native MC is low (V_1 compared to V_0 increases by 10-15%), being in the range of experimental error in different variants (Fig. 1, I), but it is reliably repeated in each individual experiment and does not depend on ionic strength (not shown). For frozen MC preparations in the medium with 100 mM KCl, the acceleration of respiration in the presence of MbO_2 is twice higher: 25-30% (Fig. 2, solid curve; Fig. 1, III). The rate of mitochondrial respiration in the presence of MbO_2 (V_1) is maximal in the medium with low ionic strength (sucrose, 250 mM), indicating complete uncoupling of the respiratory chain (Fig. 1, IV). In the case of FCCP-uncoupled native MC, there is no effect of MbO_2 on the maximum rate of O_2 uptake by MC (Fig. 1, II). In all of the mitochondrial preparations under study, the ionic strength does not influence the rate of oxygen uptake in the absence of MbO_2 (V_0).

Accelerated respiration of native and frozen liver MC in the presence of sperm whale MbO_2 was revealed by us previously [18] and confirmed later [23] for native pig

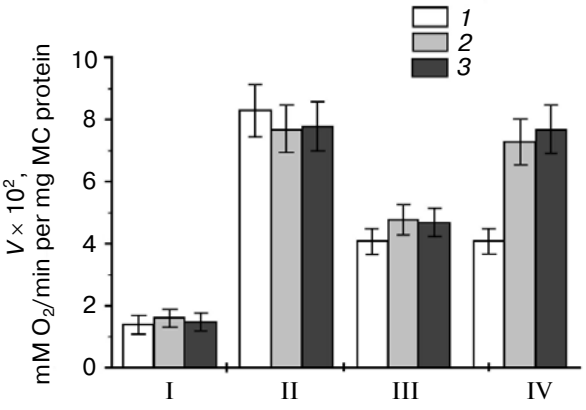

Fig. 1. Rate of O_2 uptake by rat liver MC without MbO_2 (*1*), in the presence of 0.11-0.25 mM sperm whale MbO_2 (*2*), and the rate of MbO_2 deoxygenation in the presence of MC (*3*) per 1 mg/ml of mitochondrial protein. I, native coupled MC; II, native MC uncoupled by FCCP; III, freshly frozen MC (incubation medium: EGTA, 0.5 mM; KH_2PO_4, 5 mM; Mops, 10 mM, pH 7.4; succinate, 15 mM; sucrose, 150 mM; KCl, 100 mM); IV, freshly frozen MC (the same incubation medium but containing 250 mM sucrose without KCl).

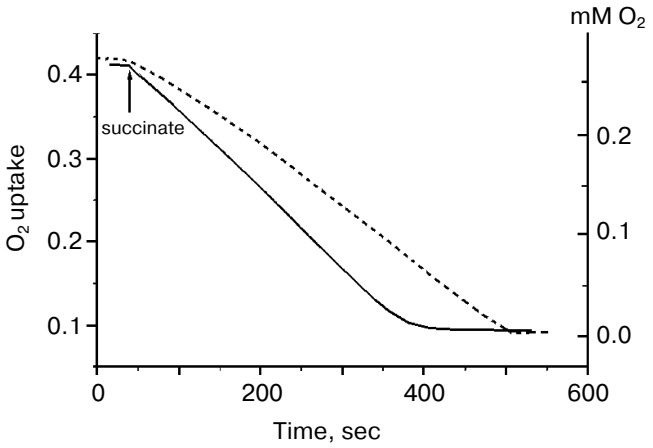

Fig. 2. O_2 uptake by freshly frozen rat liver MC in the polarographic cell without MbO_2 (dashed curve) and in the presence of 0.11-0.25 mM oxymyoglobin (solid curve). Standard incubation medium with KCl (see legend to Fig. 1), 1 mg/ml of mitochondrial protein.

heart MC in a solution of horse MbO_2 (P_{50} 0.43 mm Hg) with glutamate and malate as respiratory substrates. The effect of MbO_2 on MC respiration rate is apparently due to their interaction with the protein. Since sperm whale Mb carries a low positive charge (~2 at pH 7.4), its interaction with the negatively charged outer membrane must be maximal in a salt-free medium, which is actually observed. However, it is still unclear (and subject to further investigation) how myoglobin can influence the respiratory chain localized in the inner membrane, because the outer mitochondrial membrane is impermeable for proteins.

Figure 3a shows the result of the experiment where respiring MC are separated from MbO_2 solution by a semipermeable membrane. One can see that the protein spectrum remains unchanged even if O_2 concentration in the solution is reduced practically to 0, i.e. there is no (even partial) deoxygenation of myoglobin. If MC is added directly to the protein solution, the typical kinetics of MbO_2 deoxygenation is registered spectrophotometrically (Fig. 3b). Deoxymyoglobin formation (τ_{trans}) is observed after a certain lag period (τ_{lag}), during which the protein spectrum remains unchanged. The duration of both phases is different for various mitochondrial preparations. Lag period duration corresponds to the time when almost all O_2 is consumed from the polarographic cell (P_{O_2} < 10-15 mm Hg), so that the rate of O_2 uptake by MC V_1 determined from τ_{lag} actually coincides with V_1 found polarographically under the same conditions (table).

The rate of oxygen release from MbO_2 (V_2) is constant during the whole period of transition and does not depend on MbO_2 concentration (0-th order in myoglobin). Indeed, the reaction rate does not change on increasing the MbO_2 concentration 1.5-2.5 times (table). The MbO_2 concentration in the experiments is rather high and comparable with MbO_2 concentration of 0.3-0.5 mM in the cytoplasm of myocytes of terrestrial mammals. Hence, the concentration of MbO_2-bound oxygen is comparable with its concentration in saturated aqueous solution (0.25 mM). However, the electrode does not register any abrupt increase in O_2 concentration in solution during the transition due to the release of oxygen from MbO_2. In fact, the bend on the curve is smoother in the presence of MbO_2 at P_{O_2} < 10-20 mm Hg (Fig. 2, solid curve) than in its absence (Fig. 2, dashed curve). Therefore, this suggests that oxygen is delivered directly to MC as a result of contact with the mitochondrial membrane. The rate of oxygen release from MbO_2 in the pres-

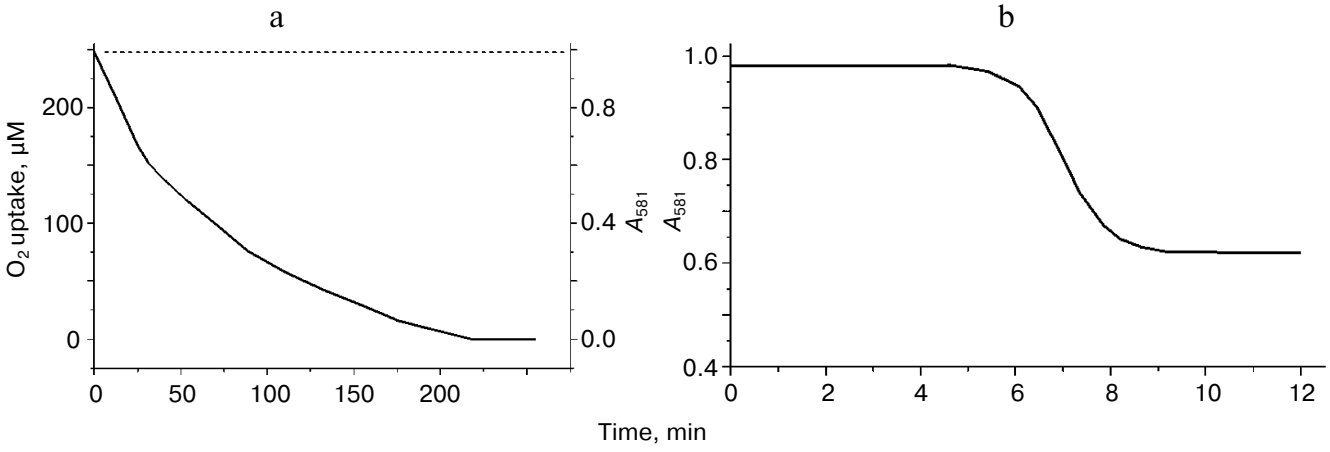

Fig. 3. a) Kinetics of O_2 uptake by frozen rat liver MC in polarographic cell (solid curve) and the variation of MbO_2 spectrum (dashed curve). Experimental conditions: MC, 0.9 ml, at mitochondrial protein concentration of 30 mg/ml, in the dialysis bag placed into a sealed cuvette with 12.5 ml of MbO_2 solution (0.11 mM). Standard incubation medium with KCl (Fig. 1). b) Kinetics of O_2 release from MbO_2 in the presence of frozen rat liver MC. MbO_2 concentration, 0.11 mM. Incubation medium and experimental conditions as in legend to Fig. 2.

O_2 uptake by rat liver MC in the presence of MbO_2 (V_1) and deoxygenation of MbO_2 in the presence of MC (V_2) according to polarographic and spectrophotometric data

Mitochondria	$[MbO_2]$, mM	$V_{1\,pol}$, μM/min	$\tau_{lag\ spectr}$, min	V_1 from $\tau_{lag\ spectr}$, μM/min	$V_{2\,spectr}$, μM/min	V_2/V_1
Native coupled	0.11 0.25	15 ± 1.5 13.5 ± 1.5	17 ± 2 19 ± 2	14.7 ± 1.5 13.2 ± 1.5	15.6 ± 1.5 14 ± 1.5	1 ± 0.05
FCCP uncoupled	0.11 0.25	73 ± 8 78 ± 8	3 ± 0.5 3.1 ± 0.5	83 ± 8 81 ± 8	77 ± 8 80.5 ± 8	0.98 ± 0.05
Frozen	0.11 0.25	50 ± 5 43 ± 5	5.5 ± 0.5 5.3 ± 0.5	45.5 ± 5 47.2 ± 5	46 ± 5 48 ± 5	1.05 ± 0.05
–"–	0.12*	–	6.5 ± 0.5	39.5 ± 4	21.6 ± 2	0.55

Note: Incubation medium same as in Fig. 1 legend.
* Sperm whale CM-MbO_2.

ence of different MC preparations measured by spectrophotometry (V_2) completely coincides with the rate of O_2 uptake (V_1) by these preparations under the same conditions (Fig. 1).

Effect of oxymyoglobin charge on rate of its deoxygenation in mitochondrial suspension. The dependence of V_1 and V_2 on pH at pH 5.6-7.6 has been studied in frozen MC (Fig. 4). It can be seen that at pH 7.2-7.6, the rates of MbO_2 deoxygenation and O_2 uptake by MC from solution coincide (V_2/V_1 ~ 1). At pH < 7.2, the rates of both processes decrease due to the lower efficiency of MC respiration, but V_2 is becoming noticeably higher than V_1 (V_2/V_1 markedly increases). The increase in V_2 compared with V_1 is probably due to the higher positive charge of the MbO_2 molecule at lower pH and its stronger interaction with the mitochondrial membrane, while diffusion of the neutral O_2 molecule does not depend on the pH of the medium.

On the contrary, in the case of CM-MbO_2 carboxymethylated on histidines (p*I* 5.2), which is charged negatively at pH 7.2-7.6, the rate of oxygen release from MbO_2 is nearly twice lower than the rate of O_2 uptake by MC (V_2/V_1 ~ 0.55) (table). Besides, CM-MbO_2, in contrast to the native protein, does not accelerate mitochondrial respiration (V_1 is not different from V_0), i.e. the negatively charged CM-MbO_2 interacts with MC much weaker than intact myoglobin.

Effects of different proteins on rate of oxygen release from oxymyoglobin in the presence of MC. It was found that monomeric proteins, e.g. apo-Mb, lactalbumin, and lysozyme, as well as tetrameric BSA at concentration of 0.25 mM, have no effect on the respiration activity (V_0) of native, frozen, or FCCP-uncoupled MC in the medium with high (KCl, 100 mM; sucrose, 150 mM) and low (sucrose, 250 mM) ionic strength, i.e. all these proteins, in contrast to MbO_2, have no uncoupling effect on the respiratory chain of MC.

In the KCl-containing medium, apo-Mb has no inhibiting effect on the rate of MbO_2 deoxygenation, because V_2 and V_1 are the same (Fig. 5, curves *1* and *2*) and V_2/V_1 ~ 1 (curve *1* on inset of Fig. 5). This indicates that the outer membrane lacks proteins or channels specific to Mb. The weakly positive apo-Mb (p*I* ~ 8.5, charge ~2 at pH 7.4), however, competes with MbO_2 for the interaction with the membrane in the salt-free medium (250 mM sucrose), where electrostatic interactions are maximal. The rate of MbO_2 deoxygenation V_2 (Fig. 5, curve *4*), however, decreases much more with increasing apo-Mb concentration than the rate of dissolved oxygen consumption by mitochondria V_1 does (Fig. 5, curve *3*, and curve *2* in inset). Noticeable decreasing of both V_1 and V_2 might be evidence that MbO_2 and apo-Mb contact with phospholipid regions of MC membrane, through which dissolved O_2 diffuses.

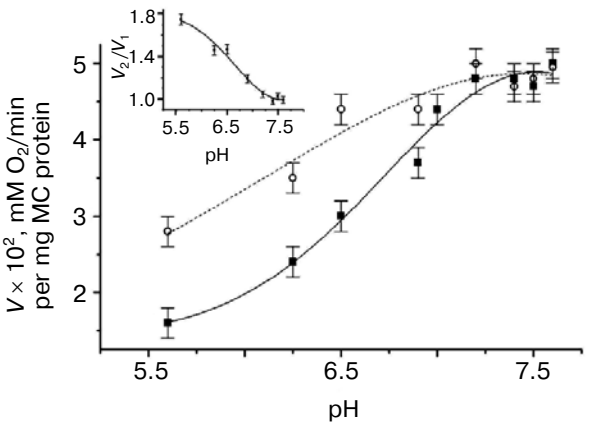

Fig. 4. pH dependence of the rate of O_2 uptake by frozen MC in the presence of MbO_2 solution, 0.11-0.25 mM (V_1, solid curve), and the rate of MbO_2 deoxygenation in the presence of MC (V_2, dashed curve). Inset: dependence of V_2/V_1 on pH. Experimental conditions as in legend to Fig. 1.

Highly positive lysozyme (protein charge 9 at pH 7.4) in equimolar concentration very strongly (almost completely) inhibits MbO_2 oxygenation even at a high ionic strength (Fig. 6, solid curve). Since the rate of O_2 uptake from solution in this case does not change noticeably even at 4-fold excess of lysozyme (Fig. 6, dashed curve), V_2/V_1 abruptly decreases (Fig. 6, inset). The strong inhibiting effect of lysozyme on V_2 suggests its effective competition with MbO_2 for binding to MC membrane and of the important role of electrostatics in this interaction.

In contrast to lysozyme and apo-Mb, the negatively charged monomeric lactalbumin (charge 8) and tetrameric BSA (p*I* 4.7) have practically no effect on the rates of both processes, V_2 and V_1, at high ionic strength (Fig. 7) and in salt-free medium (not shown), i.e. do not compete with MbO_2 for binding to mitochondrial membrane. This might be indirect evidence of the interaction of myoglobin and MC in the negatively charged phospholipid regions of the outer membrane.

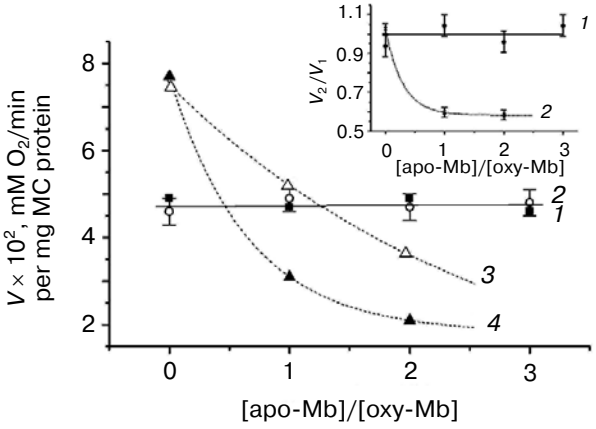

Fig. 5. Effect of apomyoglobin on rate of O_2 uptake by frozen MC in presence of MbO_2 (V_1 (*1*)) and rate of MbO_2 deoxygenation in presence of MC (V_2 (*2*)) in the medium with high ionic strength. In the medium without KCl (low ionic strength): *3*) V_1 variation; *4*) V_2 variation. In inset: ratio of rates V_2/V_1 in medium with high (*1*) and low ionic strength (*2*).

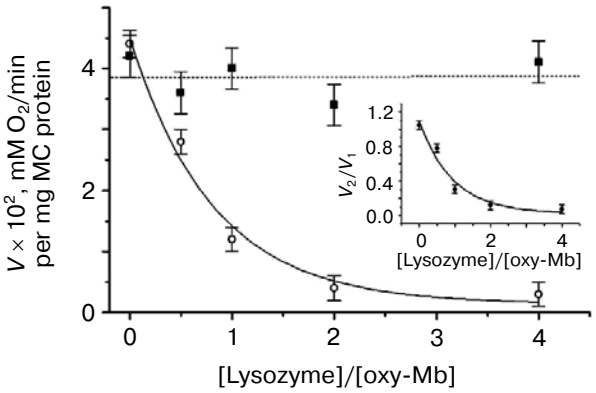

Fig. 6. Effect of lysozyme on the rate of O_2 uptake by frozen MC in presence of MbO_2 (V_1, dashed curve) and rate of MbO_2 deoxygenation in presence of MC (V_2, solid curve). In inset: dependence of V_2/V_1 on lysozyme concentration. Experimental conditions as in legend to Fig. 1.

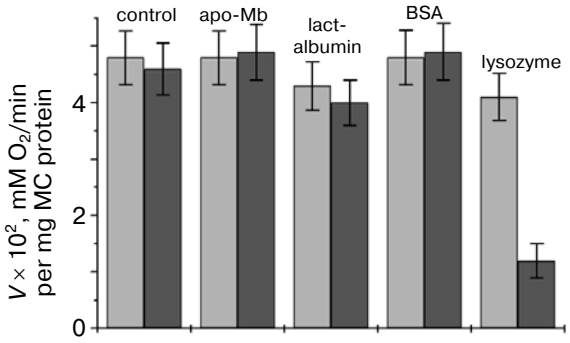

Fig. 7. Effect of different proteins (0.25 mM) on rate of O_2 uptake by frozen MC in presence of MbO_2 solution, 0.11 mM (light columns), and rate of MbO_2 deoxygenation in presence of MC (dark columns). Incubation medium and experimental conditions as in legend to Fig. 1.

DISCUSSION

Myoglobin binds fatty acids rather well, and it has even been suggested that it can function as a mobile carrier of fatty acids to MC [24, 25]. Similarly to albumin, it has the highest affinity to unsaturated fatty acids and lower affinity to saturated fatty acids. When MbO_2 is converted into the met-form, the binding is reduced by 60-70%, i.e. it depends on the ligand state of the protein [25]. Myoglobin can also interact with anionic phospholipid vesicles (liposomes) [26] and artificial and cell membranes [27, 28]. The affinity of muscle Mb (and blood Hb) to cell membranes of the bacterium *Vitreoscilla* is only 4-8 times lower than the affinity of cognate dimeric Hb [28].

The interaction of myoglobin with MC is evidenced by the fact that it is formed from apo-Mb and the heme on the mitochondrial membrane surface [29]. Besides, met-Mb is reduced by succinate in a suspension of respiring MC (no reaction proceeds without MC) [30]. In turn, MbO_2 is oxidized to met-Mb without succinate or if the respiratory chain is inhibited by antimycin A [31]. In both cases, the authors suggested that the reaction proceeds through mitochondrial cytochrome *c* on the outer membrane surface or in the intermembrane space [30, 31].

According to our data, MbO_2 deoxygenation under physiological conditions must also involve the MC membrane, because its rate, unlike the reaction rate in solution, does not depend on protein concentration (0-th order in $[MbO_2]$), is directly proportional to MC concentration,

and is completely determined by MC respiration rate ([18], present work). Indeed, the rate of MbO_2 deoxygenation for all mitochondrial states under study (spectrophotometric data) completely coincides with the rate of O_2 uptake by respiring MC (polarographic data) when the respiration accelerates (freshly frozen or FCCP-uncoupled MC) and when it slows down (native MC).

Since apo-Mb under standard conditions does not inhibit the release of oxygen from MbO_2, the MC membrane probably lacks specific proteins or protein channels interacting with myoglobin. This is in agreement with the fact that the available databases for stable protein–protein complexes [32] do not contain any for myoglobin. It is also unlikely that there are specific phospholipid regions because apo-Mb would be bound to them better than the holoprotein [28]. The revealed influence of the charges of Mb itself and other proteins on the rate of MbO_2 deoxygenation indicates that myoglobin interacts, most probably nonspecifically, with negatively charged phospholipids of the outer membrane and that electrostatics plays an important role in this interaction. In spite of the low net positive charge of myoglobin (~2 at pH 7.4), electrostatic interactions close to the negatively charged membrane surface might strengthen due to the local decrease in effective dielectric permeability and pH [33].

Optical and NMR spectroscopy, circular dichroism, and other methods have shown that the interaction of met-Mb with phospholipid membranes (vesicles), especially negatively charged ones, results in a change in Mb conformation [26]. At low phospholipid/protein ratios, the heme cavity conformation, tertiary structure, and stability of met-Mb are changed earliest, while its secondary structure and compactness are considerably maintained. We have also revealed that the rate of MbO_2 auto-oxidation sharply increases in the presence of negatively charged vesicles, and its mechanism is triggered by breakage of the H-bond between O_2 molecule and distal His64(E7) in the heme cavity [34, 35].

Minor changes in the conformation of the heme cavity of myoglobin as a result of interaction with the negatively charged membrane (electrostatic and non-electrostatic) might, in turn, substantially influence its affinity to the ligand. Indeed, it has been shown that P_{50} of myoglobin is 4-5 times higher in myocytes than in solution at 37°C and, besides, depends on the state of MC [1]. In the normal state of rat myocytes, myoglobin P_{50} (8.9 μM, 5.4 mm Hg at 37°C) is 4-fold higher than when respiration is inhibited by antimycin A and for the isolated protein in solution (2.2 μM, 1.3 mm Hg). If the respiration is 2.2-fold activated by FCCP, P_{50} value of myoglobin increases as much (up to 21 μM, 12.6 mm Hg) [36]. Myoglobin P_{50} in MC suspension is ~ 5 mm Hg at 25°C according to our data (see also [23]) and ~6.5 mm Hg at 25°C according to the data of Tang et al. [37, 38] (it is impossible to determine P_{50} more exactly because of polarographic errors at $P_{O_2} < 10$ mm Hg). Thus, as a result of interaction with MC, the affinity of myoglobin to O_2 decreases 10-12 times compared with the solution [39].

In contrast to tetrameric Hb, whose affinity to oxygen is regulated by the ligand itself and by allosteric effectors, diphosphoglycerate, protons, and CO_2, providing the necessary rate of O_2 delivery by blood, no low molecular weight compounds or metabolites that can substantially influence the affinity of Mb to the ligand are known [1]. We believe that the affinity of Mb to O_2, similar to the carrier proteins of other ligands, is regulated by its interaction with the mitochondrial membrane, which facilitates O_2 dissociation under physiological conditions of P_{O_2} 10-20 mm Hg and provides its delivery directly to MC with the necessary rate.

Consequently, in the presence of respiring MC the solution must contain at least two types of myoglobin molecules: free molecules with high affinity to O_2 and, correspondingly, low P_{50} value, as well as the Mb molecules bound to the mitochondrial membrane, with low affinity and high P_{50}. This situation can be described by the following equation:

$$\mathrm{Mb(2)} + \mathrm{O_2} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{MbO_2} + \mathrm{MC} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}}$$

$$\rightleftarrows [\mathrm{MbO_2 \cdot MC}] \underset{k'_1}{\overset{k'_{-1}}{\rightleftarrows}} \mathrm{Mb(2)} + \mathrm{MC \cdot O_2}\,, \qquad (1)$$

where k_1 and k_{-1} are the constants of O_2 binding and dissociation by myoglobin in solution (k'_1 and k'_{-1} in complex with MC); k_2 and k_{-2} are the constants of formation and dissociation of the complex. The difference of constants k'_1 and k'_{-1} from the respective constants without the upper indices is most probably due to the changed conformation of the myoglobin heme cavity as a result of protein/membrane interaction.

Accordingly, instead of the mathematical models used previously and currently for the assessment of myoglobin contribution to oxygen delivery to cells, where its affinity to oxygen is taken as constant (one pair of binding and dissociation constants), the calculations must take into account three different pairs of the constants (Eq. (1)).

We have shown ([18] and present work) that the release of O_2 from MbO_2 at physiological P_{O_2} is possible only on direct interaction with MC. In the case when respiring MC are separated from the protein solution by a semipermeable membrane, no MbO_2 deoxygenation is observed at O_2 concentration close to 0. It is also impossible to obtain deoxy-Mb in vacuum, $1\text{-}10^{-3}$ mm Hg.

Under standard conditions, the rate of MbO_2 deoxygenation (V_2) in the presence of different MC preparations completely coincides with the rate of their O_2 uptake from solution in the presence of myoglobin (V_1).

Apomyoglobin, which is similar to the holoprotein in structure, has no considerable effect on V_2 (and V_1) under standard conditions, which is evidence of the absence of specific sites of interaction with MbO_2 on the membrane.

When the positive charge of MbO_2 increases at pH < 7, the rate of MbO_2 deoxygenation becomes higher than the rate of O_2 uptake by MC from solution ($V_2 > V_1$) due to its stronger interaction with the negatively charged mitochondrial membrane. This is also confirmed by the fact that lysozyme with a high positive charge almost completely inhibits V_2 (with no effect on V_1) due to the competition with MbO_2 for binding to the membrane.

In the case of CM-MbO_2 carboxymethylated on histidines (p*I* 5.2), which is negatively charged at pH 7.4, the rate of MbO_2 deoxygenation is twice lower than the rate of O_2 uptake by MC (V_2/V_1 is 0.55), while negatively charged bovine serum albumin and lactalbumin have no influence on V_2 of native MbO_2. This is in agreement with the conclusion that MbO_2 in the course of deoxygenation most probably interacts with the outer membrane phospholipids, and electrostatics plays an important role in this process.

In cells, myoglobin functions as a typical carrier of O_2 ligand. Its affinity to oxygen, like that of protein carriers of other ligands, is regulated by the interaction with O_2-consuming MC.

The mechanism under study, in contrast to "oxygen depot" and "facilitated diffusion", can more adequately explain the physiological role of myoglobin in the functioning of myocytes under conditions of actually observed parameters (P_{O_2}, D_{Mb}, S_{MbO_2}).

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