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The effect of mitochondrial and artificial bilayer phospholipid membranes on conformation of myoglobin and its affinity for oxygen

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Abstract

We have first shown that oxygen release from MbO_2 at near-zero O_2 concentrations (p_{02}) only proceeds when interacting the protein with respiring mitochondria, but no deoxygenation of MbO_2 occurs, if they are separated from MbO_2 solution by a semi permeable membrane. The rates of O_2 uptake by mitochondria from solution in the presence of MbO_2 (V_1) and MbO_2 deoxygenation (V_2) completely coincide for different mitochondrial preparations, the native, frozen and uncoupled by FCCP, as both V_1 and V_2 are determined by respiratory activity of mitochondria. However, V_1 and V_2 reflect different processes, because they are differently affected by the proteins like lysozyme, competing with MbO_2 for binding to mitochondria. It is found that myoglobin non-specifically interacts with phospholipid sites of the outer mitochondrial membrane, while any specific proteins or protein channels for the binding to myoglobin are lacking. Physiologically active MbO_2 and not active metmyoglobin (metMb) adsorb on the surface of the artificial BLMs from neutral lecithin and negatively charged 1-palmitoyl-2-oleilphosphatidylglycerol (POPG), the adsorption activity is being much higher for negatively charged BLM and two–three fold larger for metMb compared to MbO_2. The pronounced ionic strength dependence of the binding implies significant contribution of coulombic electrostatics into the formation of myoglobin–mitochondrial complex, most probably due to local electrostatic interactions between oppositely charged groups of phospholipids (the heads) and polar myoglobin residues (invariant Lys and Arg) near the heme cavity. Interaction of metMb with POPG liposomes leads to conformational changes first of all in the heme cavity, while secondary and tertiary structures of the protein are preserved. Significant increasing of MbO_2 autooxidation rate under aerobic conditions, much more pronounced in the presence of mitochondria and negatively charged POPG liposomes, as well as some shift of MbO_2 / Mb(2) equilibrium towards ligand-free Mb(2) under anaerobic ones, both evidence in favor of decreased myoglobin affinity for O_2, induced by its interaction with phospholipid membranes, which must facilitate O_2 detachment from MbO_2 at physiological p_{02} values in the cell.

Keywords

Myoglobin, Mb, Hemoglobin, Hb, Mitochondria, MC, Oxymyoglobin, MbO_2, Oxyhemoglobin, HbO_2, Bilayer Phospholipid Membrane, BLM, Partial Pressure of Oxygen, p_{O2}, Affinity for O_2, p_{50}

1. Introduction

The most important condition for functioning of all living organisms is continuous supplying them by sufficient oxygen, access of which to metabolic processes in cells is mediated by respiratory proteins, tetrameric blood hemoglobin (Hb) and monomeric muscle myoglobin (Mb). Myoglobin is developed in red muscles in response to mitochondrial demand for oxygen, transporting it from sarcolemma to mitochondria [1-3]. Particularly high Mb concentrations are found in muscles of aquatic animals, diving for a long time for food, and also of highland species, living under O_2 deficit, which served as the basis for the hypothesis that myoglobin is the "oxygen store" for

mitochondrial cytochrome *c* oxidase. In 1940s, Hill [1] and Millikan [2] calculated that the content of myoglobin in cardiac muscles of terrestrial animals is sufficient to hold O_2 amount for a complete contraction cycle.

Myoglobin is now believed to function like hemoglobin [1-3]. Under normal conditions, when the O_2 flow from blood is sufficient for functioning mitochondrial electron transfer chains, myoglobin binds oxygen with high affinity and detaches it, when a normal blood flow is disturbed and the partial pressure of oxygen (p_{O2}) in cells falls below some crucial level (the "oxygen store" mechanism). Due to this mechanism, a high muscle performance must be maintained during muscle contractions. In skeletal muscles of many terrestrial animals, however, Mb concentration is rather small, so the stored O_2 amount is sufficient for only 8-sec contraction under maximal loadings.

Since diffusion processes play very important role in the O_2 transport and consumption systems, another hypothesis was also developed that myoglobin can facilitate O_2 diffusion in the cell due to reversible combination of the protein with oxygen and translational diffusion of MbO_2 molecules to mitochondria (the myoglobin-facilitated oxygen diffusion mechanism) [4-7]. Wittenberg, who was the first reported evidence for it [4], and other authors believe that it is the intracellular transport of oxygen from sarcolemma to mitochondria rather than its deposition, is the main function of myoglobin in muscles. Many experimental and theoretical studies are devoted to the phenomenon of the O_2 diffusion facilitated by the respiratory proteins, HbO_2 or MbO_2 [3]. For example, the rate of labeled $^{18}O_2$ transfer through porous membranes filled with MbO_2 solution, was shown to 15-fold accelerate compared to that with metMb solution [8]. However, the physiological significance of myoglobin-facilitated O_2 diffusion is a subject of hard controversy since the Wittenberg's time until now. While some authors make conclusions in favor of significant contribution of this mechanism in supplying cells with oxygen, while the others deny it completely [9-13].

At present, earlier accepted mechanisms of myoglobin functioning and even its role in O_2 delivery to mitochondria are revised [14-18]. The reason is primarily the appearance of new experimental techniques, allowing to accurately determin many cell parameters *in vivo*, which was not available previously, and also the development of genetic engineering. It appeared, for example, that in addition to O_2 storing, myoglobin can regulate the bioactive NO level in cells, playing nitrite reductase function, which is very important for cardiac energetic and antioxidant protection of the heart [19-22]. Besides, myoglobin may act as a mobile carrier of fatty acids important for maintaining their concentration as main substrates of cardiac metabolism [23-25].

What is the true mechanism of myoglobin functioning and its role in O_2 delivery to mitochondria? To elucidate the problem is very important not only for fundamental molecular biology and biochemistry, but also for solving very many applied aspects related to providing best oxygen conditions in cell growth cultures. To decide, for example, if the genes, expressing myoglobin, have to be introduced into the culture (which is very expensive), it is necessary to assess properly, using mathematical modeling, the contribution of myoglobin in supplying cells with oxygen [26].

2. How Does Myoglobin Work

2.1. The "Oxygen Store" Mechanism of Myoglobin Functioning

This mechanism assumes that myoglobin must act in accordance with its oxygenation curve in solution (Fig. 1), i.e. the protein oxygenation degree *versus* O_2 concentration in solution, usually expressed by p_{O2} value. The oxygenation of myoglobin is described by the hyperbolic curve in the very low p_{O2} region (Fig. 1, curve 1), while Hb oxygenation is presented by the sigmoid curve in the moderately low p_{O2} region (Fig. 1, curve 2), implying that Hb affinity for O_2, corresponding to the p_{O2} value at 50 % oxygenation (p_{50}), is regulated by the ligand. The rate of Mb oxygenation is more than six times higher compared to that of Hb, and its O_2 affinity is 100-fold higher, which determines the physiological role of myoglobin as the oxygen store in muscles of mammals [3, 27]. At p_{O2} in venous blood, equal to 40 mm Hg, the oxygenation degree of Hb is only 60%, whereas Mb is oxygenated almost completely (Fig. 1).

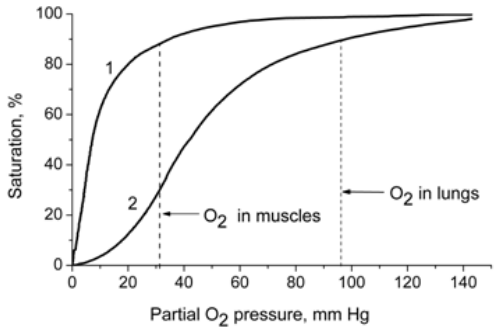


Fig. 1. *The oxygenation curves of muscle myoglobin, pH 7.6 (1) and blood hemoglobin, pH 6.8 (2).*

The affinity of tetrameric Hb for oxygen is regulated also by diphosphoglycerate, protons and CO_2, providing the optimal rate of O_2 delivery from blood [27, 28]. At the same time, no low molecular weight compounds or cellular metabolites capable to affect Mb affinity for O_2, moving its oxygenation curve toward higher p_{O2} values, are known. As p_{50} values of different animal myoglobins are very close and equal to 0,5–1,0 mm Hg at room temperature (2,0 - 2,75 mm Hg at 37^0C) [3, 27], the functioning of myoglobin in accordance with the "oxygen store" mechanism can be effective only at very low p_{O2} in the cell about 0,5–3,5 mm Hg. Respectively, a low level of the protein saturation by oxygen (S_{MbO2}) should be observed in working muscles (10-30 %). However, p_{O2} values in hardly working heart are estimated now from 15 to 25 mm Hg and S_{MbO2} values defined by NMR technique do not fall below 85%, amounting to 92% of the total pool of the

protein [10, 11, 29]. Thus, within the cell, both extremely low p_{O2} values and low S_{MbO2}, i.e. high Mb(2) concentrations are lacking, which makes the "oxygen store" mechanism of the myoglobin functioning very improbable.

Moreover, the reversibility of the Mb oxygenation curve (Fig. 1, curve 1) is now very doubtful, because we failed to obtain ligand free Mb(2) directly from MbO_2 even at the deep vacuum below 1 mm Hg [30]. At the same time, Hb(2) can be easily obtained from HbO_2 at 5-10 mm Hg in accordance with its oxygenation curve (Fig. 1, curve 2). Note, that Mb(2) is usually prepared from metMb or MbO_2 using chemical reluctant, mostly sodium dithionite, or very seldom, NADH with illumination by visible light in vacuum in the presence of photosensitizes [27, 31]. But these reducing substances react with both the dissolved oxygen, removing it from solution, and directly with the liganded O_2 bound to the heme Fe atom.

Since the equilibrium in the reaction of deoxymyoglobin with oxygen is strongly shifted towards the liganded MbO_2 (Equation 1), the oxygenation curve usually receive by association of Mb(2), prepared from metMb or MbO_2 using dithionite, with known O_2 aliquots, the ratio of the liganded and ligand free forms of the is being measured spectrophotometrically [3, 27, 32]:

$$Mb(2) + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MbO_2 \qquad (1)$$

where k_1 and k_{-1} are the kinetic O_2 binding and dissociation constants in solution.

The binding constant k_1 is determined with rapid methods, adding some O_2 to Mb(2) solution. The dissociation constant k_{-1} is determined in the same way by adding some dithionite to MbO_2 solution, or with potassium ferricyanide that irreversibly oxidizes MbO_2 to metMb, or, finally, displacing liganded O_2 by carbon monoxide (CO) [27]. As studies of the O_2 dissociation properties of myoglobin are not numerous, it cannot be excluded that the oxygenation curve of myoglobin (Fig. 1) really has a hysteresis, and O_2 dissociation from MbO_2 is possible only at much lower p_{O2} values than known from the literature.

2.2. The Myoglobin Facilitated O_2 Diffusion Mechanism

On the one hand, it is obvious that the rates of lateral and rotational diffusion of large protein molecules are much smaller than those of small O_2 molecules, so that myoglobin cannot compete with them in consumption of mitochondria. On the other, an argument in favor of the facilitated diffusion mechanism is the fact that concentration of O_2 bound to myoglobin in working heart of all warm-blooded animals is in thirty or even more times higher than concentration of free O_2 in the cell [4-7]. The key points for the theory of the facilitated diffusion phenomenon are high concentrations of myoglobin in the cell, high rates of its lateral diffusion (D_{Mb}), as well as large gradients of MbO_2 concentration, which corresponds to low intracellular S_{MbO2} under great muscle loads [9, 12]. Thus, the "facilitated diffusion" mechanism can be effective also at very low p_{O2}, and besides, its contribution to supplying cells with oxygen depends on the diffusion capacity and gradients of MbO_2 concentration.

As already mentioned, both extremely low p_{O2} and high gradients of MbO_2 concentration, are absent in working heart [10, 11, 29]. Also, diffusion coefficients of myoglobin in intact skeletal muscles ($1.2x10^{-7}$ and $2.1x10^{-7}$ cm^2/sec at 22° and 37 °C, respectively) appeared to be 5-10 times lower than D_{Mb} values used in calculations of contribution of the "facilitated diffusion" in supplying the cells with oxygen, so that, at p_{O2} <13 mm Hg, this contribution is only 1,5-4% and increases slightly at p_{O2} > 13 mm Hg [12, 33, 34]. The calculations with D_{Mb} for mouse heart [35], which is about 3 times higher than D_{Mb} in skeletal muscle, show nevertheless that at 0.2 mM concentration of MbO_2 and p_{O2}, equal to 1.8 mm Hg, contributions of the free and bound oxygen are similar and only at lower p_{O2}, the latter begins to dominate.

Thus, the effective functioning of myoglobin according to earlier accepted the "oxygen store" and "facilitated O_2 diffusion" mechanisms is questionable for today. Both mechanisms are formulated in the framework of homogenous thermodynamics and kinetics in solution, assuming no interaction of myoglobin with any cellular structures or metabolites. Hence, the myoglobin affinity for O_2 is taken constant in all model calculations of its contribution in supplying cells with oxygen [10, 13, 26].

2.3. Oxymyoglobin Interacts with Mitochondrial Membrane During Deoxygenation Process

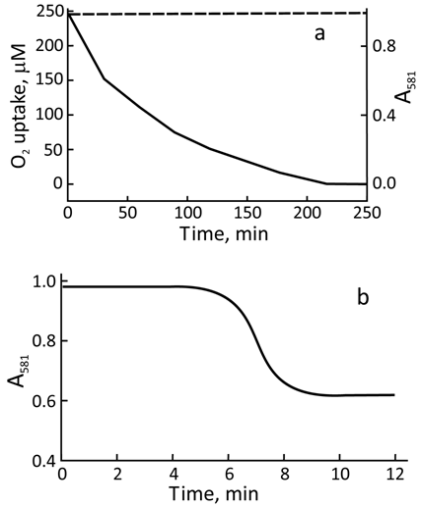


Fig. 2. *a) Kinetics of O_2 uptake by frozen rat liver mitochondria (the solid line) and variation of sperm whale MbO_2 absorption spectrum (the dashed line). Experimental conditions: mitochondria, 0.9 ml, at mitochondrial protein concentration of 30 mg/ml, are put in the tightly packet dialysis bag placed into a sealed polarographic cuvette with 12.5 ml of MbO_2 solution (0.07 mM). Incubation medium: EGTA, 0.5 mM; KH_2PO_4, 5 mM; Mops, 10 mM, succinate, 15 mM; sucrose, 150 mM; KCl, 100 mM, pH 7.4. b) Kinetics of O_2 release from MbO_2 in the presence of frozen rat liver mitochondria. MbO_2 concentration, 0.07 mM; mitochondrial protein concentration of 1 mg/ml. Incubation medium and experimental conditions as in a).*

We first showed that O_2 release from MbO_2 at physiological p_{O2} values proceeds only upon direct contact of the protein with mitochondria [30, 35]. No MbO_2 deoxygenation occurs even at near-zero O_2 concentration (Fig. 2, a), when respiring mitochondria are separated from MbO_2 solution by a semi permeable membrane, being put in the tightly closed dialysis bag. But if after several hours of incubation at room temperature, they are added to the MbO_2 solution, Mb(2) absorption spectrum is rapidly (within several minutes) registered. When respiring mitochondria are initially added to MbO_2 solution, the kinetics of MbO_2 deoxygenation is observed after some time (τ_{lag}), when MbO_2 spectrum does not change (Fig. 2, b). Lag period corresponds to the time needed for O_2 consumption by mitochondria from solution, which is measured in the polarographic cell under the same conditions. Thus, the rates of O_2 uptake by mitochondria from solution in the presence of MbO_2 (V_1) determined both polarographically and found spectrophotometrically from τ_{lag} are virtually identical (Table 1).

Table 1. *Oxygen uptake by rat liver mitochondria in presence of MbO_2 (V_1) and deoxygenation of MbO_2 in the presence of mitochondria (V_2) according to polarographic and spectrophotometric data. Incubation medium: MOPS, 10 mM, pH 7.4; succinate, 15 mM; sucrose, 150 mM; KCl, 100 mM, EGTA, 0.5 mM; KH_2PO_4, 5 mM; protein concentration of mitochondria 1 mg/ml.*

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

* – sperm whale Mb carboxymethylated at histidines residues by bromoacetate (CM-MbO_2, *pI* 5.2).

The rate of oxygen release from MbO_2 measured spectrophotometrically (V_2) is constant during all transition period (τ_{tr}) and does not depend on MbO_2 concentration (the 0-th order in myoglobin). Indeed, the reaction rate does not change with increasing MbO_2 concentration in 1,5-2,5 times (Table 1). Lowering the reaction order in comparison with the reaction of 1-th order for MbO_2 deoxygenation in solution (Equation 1) is inherent for the process involving membranes [31], pointing to that the number of surface sites on mitochondrial membrane, interacting with MbO_2, much less its concentration. In favor of this is the fact that V_2 rise linearly with increasing concentration of mitochondria in the suspension (Fig. 3).

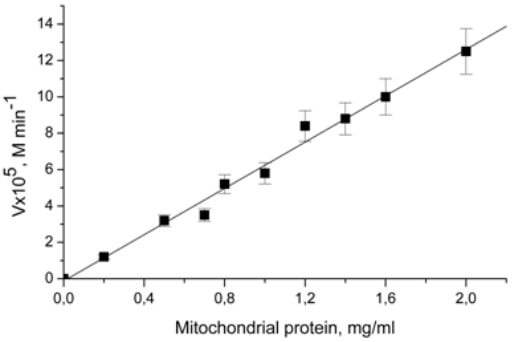


Fig. 3. *The dependence of deoxygenation rate sperm whale oxymyoglobin on the amount of mitochondria in the MbO_2 solution (0.24 mM). Mitochondrial protein concentration of 1 mg/ml, incubation medium as in Fig. 2, a.*

The rate of O_2 uptake by mitochondria from solution (V_0) increases in the presence of MbO_2 (but not of metMb), $V_1 > V_0$ (Fig. 4). In all experiments, MbO_2 concentration was rather high and comparable with it of 0,3-0,5 mM in myocyte cytoplasm of terrestrial animals. Hence, the concentration of O_2 bound to myoglobin is comparable with its 0,25 mM concentration in saturated aqueous solution. However, the electrode does not register any abrupt increase in O_2 concentration in solution during oxygen release from MbO_2 at p_{O2} <10-20 mm Hg, which suggests that bound O_2 is delivered directly to the mitochondrial membrane.

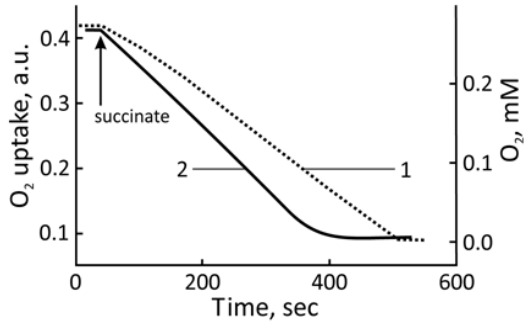


Fig. 4. *Oxygen consumption by freshly frozen rat liver mitochondria in the polarographic cell without MbO_2 (the dashed curve) and in the presence of 0.11–0.25 mM sperm whale oxymyoglobin (the solid curve). 1 mg/ml of mitochondrial protein, standard incubation medium with KCl (see legend to Fig. 2, a).*

The uncoupling effect of sperm whale MbO_2 firstly revealed by us [30] was also confirmed later for horse MbO_2 and mitochondria from pig heart with glutamate and malate as respiratory substrates [36]. For native mitochondria, the effect of MbO_2 is small, composing only 10-15% of V_0, though it is repeated in all experiments. For frozen mitochondria, the uncoupling effect is twice as large, 25–30% of V_0, in the respiring medium with 100 mM KCl, reaching maximal values in the salt-free medium [35]. The effect is completely lost, if mitochondria are uncoupled by FCCP, i.e., MbO_2 has no influence on their maximal O_2 consumption rate. Note that

for all mitochondrial preparations studied, ionic strength has no effect on V_0. The uncoupling effect must be obviously due to the interaction of MbO_2 with mitochondrial membrane, but its nature remains unclear, since the outer membrane of mitochondria is impermeable to proteins. It has to be studied in what way MbO_2 might affect the respiratory chain located in the inner mitochondrial membrane.

It is very important that for different preparations of mitochondria, native, frozen and uncoupled by FCCP, MbO_2 deoxygenation rate measured spectrophotometrically, V_2, completely coincides with the rate of O_2 uptake by mitochondria from solution in the presence of MbO_2, V_1, since V_2 is evidently fully determined by respiring activity of mitochondria, both when it is accelerated or slowing down (Table 1).

Thus, only p_{02} falling in the cell is not enough for O_2 cleavage from MbO_2 (the "oxygen depot" mechanism does not work), but MbO_2 deoxygenation should be a process, proceeding with the active participation of the mitochondrial membrane. The result of the myoglobin - mitochondrial interaction might be reduced myoglobin affinity for the ligand that must facilitate MbO_2 deoxygenation at physiological p_{02} values.

2.4. The Electrostatic Nature of the Myoglobin - Mitochondrial Interaction

Myoglobin is obviously able to interact with mitochondria, as it is formed from apoMb and the heme on the surface of outer mitochondrial membrane [37]. It is also shown that metMb can be reduced to deoxyMb by succinate in a suspension of respiring mitochondria (no reaction proceeds without mitochondria) [38]. In turn, without succinate or if the respiratory chain of mitochondria is inhibited by antimycin A, MbO_2 oxidizes to metMb [31]. In both cases, the reaction most probably proceeds through mitochondrial cytochrome *c*, which could react with myoglobin at the surface of the outer membrane or within the intermembrane space.

2.4.1. Competitive Effects of Different Proteins on the Rate of MbO_2 Deoxygenation in Suspension of Mitochondria [35]

To verify the presence on the mitochondrial membrane of some proteins, protein channels or phospholipid sites, capable to specifically interact with myoglobin, the competitive effect of different proteins on the rate of MbO_2 deoxygenation in the presence of rat liver mitochondria, V_2, has been studied. For this purpose, apomyoglobin (apoMb) structurally homologous to the holoprotein but not binding O_2 was used. The outer mitochondrial membrane, 50% of which is occupied by proteins, is negatively charged. So, to assess the role of coulombic electrostatics in the myoglobin – mitochondria interaction, the effect of negatively and positively charged proteins, monomeric lactalbumin (pI 4.4), tetrameric of bovine serum albumin (pI 4.7) and egg lysozyme (pI 11), on V_2 was also studied. At last, the deoxygenation rate of chemically modified sperm whale CM-MbO_2 (pI 5.2), carboxymethylated at all surface histidines, was determined. Control experiments demonstrated that all these proteins at 0.25 mM concentration did not affect the respiratory activity (V_0) of native, frozen and FCCP-uncoupled mitochondria in the high and low ionic strength medium, i.e. unlike native MbO_2, they make no uncoupling effect.

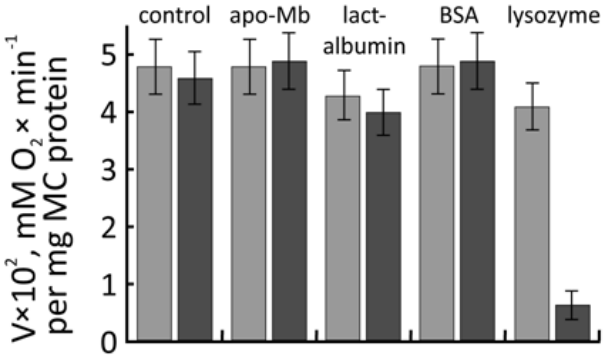


Fig. 5. *The effect of different proteins (0.25 mM) on the respiration of frozen mitochondria (V_1) in the sperm whale MbO_2 solution (light columns), and the rate of MbO_2 deoxygenation (V_2) in the presence of mitochondria (dark columns). Concentration of MbO_2 is 0.11 mM; 1 mg/ml of mitochondrial protein; incubation medium and experimental conditions as in Fig. 2, a.*

Apomyoglobin does not inhibit the rate of MbO_2 deoxygenation under standard conditions, because V_2 and V_1 are nearly equal (Fig.5), pointing that the mitochondrial membranes lack any specific proteins or protein channels for interaction with myoglobin. This is in agreement with the fact that available databases for stable protein–protein complexes [39] do not contain any for myoglobin. It is also unlikely that any myoglobin-specific phospholipids sites exist on the membrane, as more hydrophobic apoMb would associate with them much better than the holoprotein [40].

As (Fig.5) shows, negatively charged lactalbumin and BSA also have virtually no effect on V_2 (and V_1), implying that they do not compete with MbO_2 for binding to the mitochondrial membrane. In contrast, highly positive lysozyme (the net protein charge is +9 at pH 7.4) strongly inhibits the MbO_2 deoxygenation (of ~70%) even at equimolar concentration of lysozyme), while the rate of O_2 uptake by mitochondria from solution does not change noticeably even at 4-fold excess of lysozyme. The strong inhibiting effect of lysozyme on V_2 (without influencing V_1) is convincing evidence of its effective competition with MbO_2 for binding to the mitochondrial membrane. Under the same conditions, the rate of O_2 release from CM-MbO_2 is nearly twice lower than the rate of O_2 uptake by mitochondria from solution, $V_2/V_1 \sim 0.55$ (Table 1). In contrast to the native protein, CM-MbO_2 does not accelerate the mitochondrial respiration (V_1 does not differ from V_0), i.e. the negatively charged CM-MbO_2 evidently interacts with mitochondria much weaker than intact MbO_2 does.

Thus, under physiological conditions, O_2 consumption of mitochondria from solution and MbO_2 deoxygenation represent different processes, the rates of which are differently affected by proteins competing with myoglobin for binding to the mitochondrial membrane. The findings obtained also point

to lacking of any specific sites on the mitochondrial membrane for binding of myoglobin. At the same time, they indicate that myoglobin most probably nonspecifically interacts with negatively charged phospholipids of the outer membrane, electrostatics playing very important role in the interaction. Note that electrostatic interactions near to a negatively charged membrane surface might markedly strengthen due to the local decrease of effective dielectric permeability and pH [41].

2.4.2. Determination of Characteristic Parameters of Myoglobin - Mitochondrial Interaction by Fluorescent Method [42]

As the heme group of myoglobin effectively quenches emission of different donors, quenching the own tryptophan and flavin fluorescence of mitochondria by myoglobin was studied. Also, quenching the fluorescence of two membrane probes, 1-anilinonaftalino-8-sulfonate (1,8-ANS) and 5-[3-γ-sulphopropyl-2(3H)-benzoksazolidin)-2-butenilidin]-1,3-dibutyl-2-thiobarbituric acid (merocyanine M 540), embedded into the mitochondrial membrane was investigated. Both fluorescent probes are widely used in studies of natural and artificial membranes, 1,8-ANS is shown to can bind to both proteins having hydrophobic domains and phospholipids, while M 540 is selective only to phospholipids [43]. The physiologically active MbO_2 and not active oxidized metMb unable to bind oxygen were used as the quenchers in the pH range 6–8 at different ionic strengths of the medium.

The quantum yield (q) of 1,8-ANS fluorescence, which is only 0.004 in water (maximum emission is at 520 nm), increases sharply (ten times) upon binding to lipids or hydrophobic regions of proteins, the spectrum maximum being shifted to shorter wavelengths. Most researchers suppose that 1,8-ANS associates with specific phospholipid clusters of mitochondria and submitochondrial particles, which answers to relatively low degree of fluorescence polarization of the probe (P = 0,19), whereas it is about 2 fold higher (P = 0,3 - 0,4) in the case of 1,8-ANS incorporation into hydrophobic cavities of proteins [43]. Upon 1,8-ANS binding to mitochondria and lipid mono- and bilayers q varies from 0,1 to 0,3 and the fluorescence lifetime (τ_{fl}) is 6-9 ns, whereas 1,8-ANS incorporation in hydrophobic heme cavity of apoMb, for example, leads to q equal to 0,9 and τ_{fl} to 16,5–18,7 ns [43, 44].

At high 1,8-ANS concentrations in solution (up to 1 mM), there are two types of binding sites of the probe on the mitochondrial membrane, with the high and low affinity (k_{dis} is $2.5x10^{-7}$ and $1.8x10^{-4}$ M, respectively). But at low, micro molar 1,8-ANS concentrations, only the high affinity sites are saturated, the number of which is 4 nM per 1 mg of mitochondrial protein [43, 45]. In this case, 1,8-ANS fluorescence spectra meet only the bound probes and the membrane structure does not significantly alter.

Merocyanine M 540 is the representative of a large family of polyene dyes that are widely used as fluorescent membrane probes [43]. Intercoversion between three forms of M 540 occurs upon its binding to phospholipids: the fluorescent water-monomer (emission at 565 nm, the fluorescent monomer associated with the membrane (emission at 580 nm), and M 540 dimer bound to membrane which does not fluoresce [46]. When using low concentration of M 540, <1 μM , when all the dye is linked, no dimerization is observed.

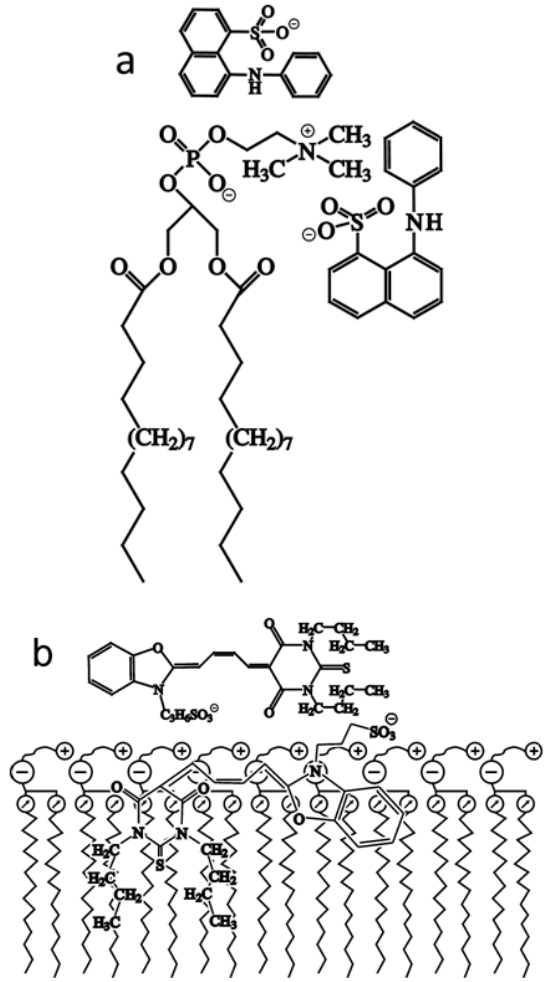


Fig. 6. *The proposed disposition of 1,8-ANS (a) and merocyanine 540 (b) in a bilayer phospholipid membrane.*

The proposed location of 1,8-ANS and M 540 in a phospholipid bilayer is shown on Fig. 6,a,b [43, 45, 47]. Since the charged sulfonic groups (pK_a is –1) should remain on the membrane surface, 1,8-ANS molecule can not sink deeply into bilayer, being located in such a way that one surface of the phenyl ring is adjacent to phospholipid glycerin sites, and the other is in contact with water (Fig. 6, a). The molecule of M 540 consists of unsaturated hydrocarbon chain containing two ring systems and also carries a negative charge on the sulfonic group (Fig. 6, b). Therefore, M 540 is localized in the intermediate area of polar heads of the phospholipid bilayer with anionic sulfonic group oriented in the direction of a more polar outer surface of the heads. The rest part of the godlike dye is located between the ether links of the phospholipid, anchoring the two butyl groups in the hydrocarbon chains [47]. Both 1,8-ANS and M 540 with charged sulfonic groups are not able to easily penetrate through the mitochondrial

membrane, at least, they do not penetrate during the experiment) [45, 48].

We have found that both tryptophan and flavin fluorescence of mitochondrial suspension is not quenched by neither MbO_2 nor metMb in the pH 6–8 range [42]. The heme group of myoglobin is not localized on the protein surface, but embedded into the protein rather deeply. Therefore, the dynamic quenching of the donor emission by myoglobin is not possible. Energy transfer from the donor molecule to the heme group of myoglobin can occur only in the complex with τ_{fl} exceeding that of the donor (the static quenching). Crucial distance of the inductive resonance energy transfer (the Förster radius) in the pairs of the myoglobin heme - tryptophan and the heme - flavin is quite large, accounting for~ 3,7 and ~ 5,0 nm, respectively [43]. Since flavins are part of the electron transport chains located in the inner membrane of mitochondria, the absence of the flavin fluorescence quenching indicates that both MbO_2 and metMb do not contact with the inner mitochondrial membrane, because myoglobin (like other proteins) is not able to penetrate through the outer membrane, thus evidencing the integrity of investigated mitochondria.

The absence of quenching the tryptophan fluorescence of mitochondria by MbO_2 and metMb implies that both myoglobins do not form any stable quenching complexes with the proteins, occupying about 50% of the outer membrane surface. It was shown earlier that, if the fluorescent probe is located in the membrane, but some protein is not bound with it, no energy transfer from protein tryptophans to the probe proceeds [43]. The fluorescence of two invariant tryptophans of myoglobin itself, Trp7(A5) and Trp14(A12), located at a distance, respectively, 2,15 and 1,5 nm from the heme, is almost completely quenched by the heme complex (q of the Trp fluorescence in holomyoglobin is only 1-5% of that in water) [44, 49].

On the contrary, both MbO_2 and metMb effectively quench the fluorescence of 1,8-ANS and M 540 bound to mitochondria (Fig. 7, a,b and Fig. 8, a,b). Although the quenching extent of 1,8-ANS and M 540 fluorescence by both myoglobins is approximately the same (within the experimental error), the binding constant (K_m) of metMb is about 1,5 fold higher, than that for MbO_2 (Table 2). The relatively small quenching extent, 30 ± 10%, of the fluorescence of 1,8-ANS and M 540, do not penetrating the outer membrane, can possibly be explained by the fact that not all bound probes are able to form efficient complexes with the quencher because of heterogeneity of their binding sites and (or) improper mutual orientation of the donor and acceptor. Note that K_m values found for each MbO_2 and metMb as the quencher do not depend on pH in the pH 6–8 range, because they do not differ at pH 6.4 and 7.4.

When quenching 1,8-ANS and M 540 fluorescence by metMb is performed at various KCl concentrations in the 10–150 mM interval, K_m values decreased with increasing ionic strength (Fig. 9, a,b), indicating the important role of electrostatic interactions in the formation of the myoglobin-mitochondria quenching complex. Note that K_m values determined at pH 7.4 and 6.4 for different ionic strengths are identical within experimental error (Table 3), i.e. do not depend on the protein total charge that varies in the pH 6–8 range.

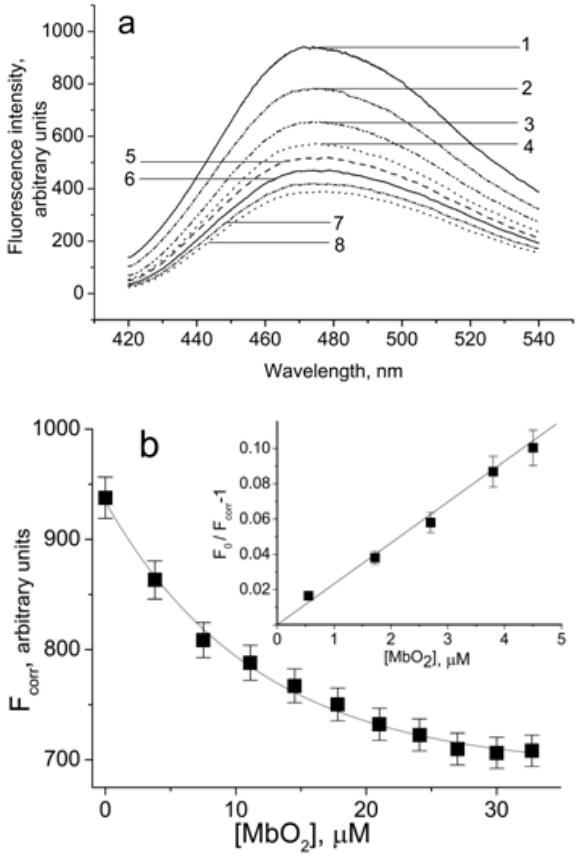
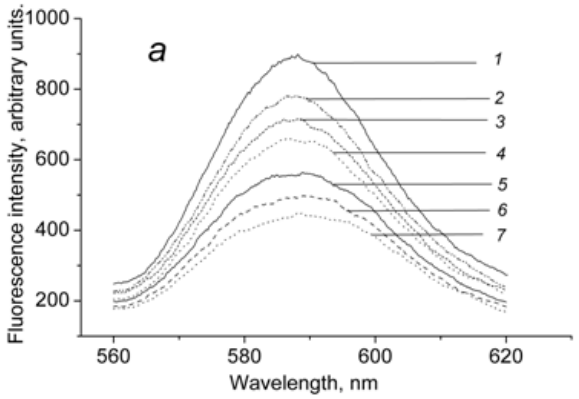


Fig. 7. *(a) The quenching of the fluorescence of mitochondria-associated 1,8-ANS probe by sperm whale oxymyoglobin. Spectra 1–8 correspond to MbO_2 concentrations of 0, 3.8, 7.5, 11.1, 14.5, 17.8, 21.0 and 24.1 μM, respectively. Excitation wavelength at 360 nm, emission spectrum maximum is 470 nm, monochromator slit widths (for excitation and emission) are 5 × 5 nm. All measurements were carried out on a Perkin-Elmer MPF-44B spectrophotometer in a quartz cell, 2 × 2 mm, to diminish the light scattering by mitochondria. Concentration of mitochondrial protein is 0.2 mg/ml. Incubation medium without succinate (10 mM Tris-HCl buffer, sucrose, 250 mM; EGTA, 0.5 mM; KH_2PO_4, 5 mM, pH 7.4). (b) The dependence of the corrected fluorescence intensity of 1,8-ANS in its spectral maximum (F_{corr}) on MbO_2 concentration, pH 7.4. At the inset: the determination of the constant of MbO_2 binding with mitochondria in the quenching complex.*



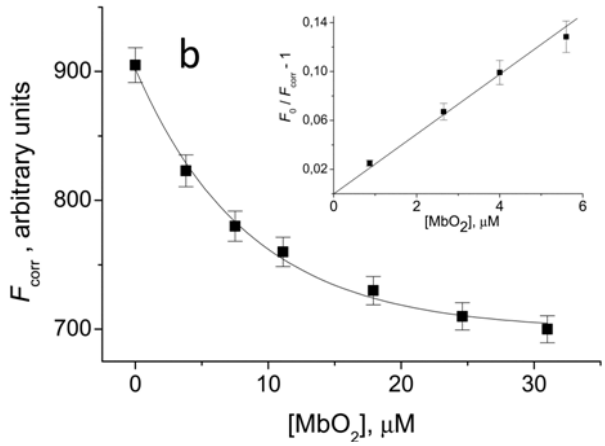


Fig. 8. *(a) The quenching of the fluorescence of mitochondria-associated M 540 probe by sperm whale oxymyoglobin, pH 7.4. 1–7 – MbO_2 concentrations are 0, 3.8, 7.5, 11.1,17.9, 24.6, 31 μM, respectively. The measurements were carried out on a Perkin-Elmer MPF-44B spectrophotometer in a quartz cell, 2 × 2 mm. Excitation wavelength is 540 nm, the spectral maximum at 585 nm, monochromator slit widths, 6 × 5 nm. Incubation medium without succinate as in Fig.7, a). (b) The corrected fluorescence intensity of M 540 in the spectral maximum vs. MbO_2 concentration. At the inset: determination of the constant of MbO_2 binding with mitochondria in the quenching complex.*

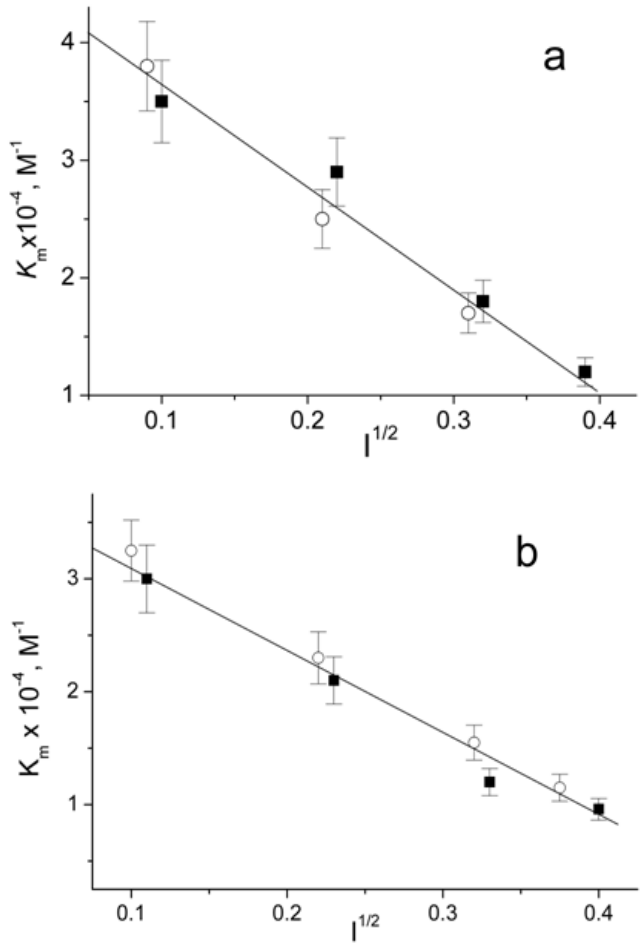


Fig. 9. *The dependence of the binding constant of sperm whale metMb with mitochondria in the quenching complexes with 1,8-ANS (a) and M 540 (b) on ionic strength. Light symbols correspond to the data obtained at pH 6.4 and dark symbols – at pH 7.4. Experimental conditions are the same as in Fig.7, a.*

Thus, the fluorescence of both 1,8-ANS and lipid probe M 540, is equally well quenched by MbO_2 and metMb, the quenching parameters (quenching degrees and K_m values of the quencher) being very close (Tables 2 and 3). This favors (*i*) the location of both probes in phospholipid regions of the outer mitochondrial membrane and (*ii*) the binding of MbO_2 and metMb to these areas. The τ_{fl} values of ANS and M 540 quenching complexes with myoglobin should exceed, respectively, 6-9 ns [43] and ~2 ns [46].

The clear dependence of K_m on ionic strength (Table 3) implies a significant contribution of electrostatic interactions into formation of the myoglobin–mitochondrial complex. The net charge of myoglobin molecule is shown not to influence the MbO_2 and metMb binding to mitochondria. As metMb affinity for mitochondria is 1,5 fold higher than that of MbO_2 (Tables 2 and 3), the effect of ionic strength must be due to local electrostatic interactions between myoglobin and mitochondria, as the difference between MbO_2 and metMb cannot be explained by ligand-induced conformational changes in the protein structure. For differently liganded myoglobins, they are very small and occur mainly in the distal part of the heme cavity, where the ligand binds [39]. Most obviously, the difference in the electronic and charge state of the heme complex in MbO_2 and metMb play major role here, namely, between the diamagnetic neutral ferrous heme in MbO_2 (the Fe ligand is O_2 molecule) and the paramagnetic charged ferric heme in metMb (charge +1 and the Fe ligand is H_2O molecule). So, local electrostatic interactions might involve oppositely charged polar groups of phospholipids (the heads) and myoglobin residues, surrounding the heme cavity (Fig. 10, a,b).

Table 2. *Quenching the mitochondria–associated 1,8-ANS and M 540 fluorescence by sperm whale oxy- and metmyoglobins at pH 6-8 (incubation medium without succinate as in Fig. 7,a).*

Ligand form of myoglobin	pH 7.4		pH 6.4	
	K_m×10^{-4}, M^{-1}	Quenching degree, %	K_m×10^{-4},M^{-1}	Quenching degree,%
	1,8-ANS			
MbO_2	2.3 ± 0.3	24 ± 3	2.7 ± 0.4	20 ± 3
metMb	3.5 ± 0.4	31 ± 3	3.8 ± 0.4	21 ± 3
	M 540			
MbO_2	2.5 ± 0.3	23 ± 3	2.8 ± 0.3	17 ± 3
metMb	3.1 ± 0.4	37 ± 4	3.2 ± 0.4	17 ±3

Table 3. *Constants of the sperm whale metMb binding with mitochondria at pH 6-8 and ionic strength from 10 to 150 mM KCl (experimental conditions as in Fig.7, a).*

Ionic strength, mM	**K_m×10^{-4}, M^{-1} from M 540 fluorescence quenching**		**K_m×10^{-4}, M^{-1} from 1,8-AHC fluorescence quenching**	
	pH 6.4	**pH 7.4**	**pH 6.4**	**pH 7.4**
0.01	3.0 ± 0.3	3.1 ± 0.3	3.8 ± 0.4	3.5 ± 0.4
0.05	2.2 ± 0.2	2.1 ± 0.2	2.5 ± 0.3	2.8 ± 0.3
0.1	1.3 ± 0.2	1.25 ± 0.2	1.75 ± 0.2	1.8 ± 0.2
0.15	1.1 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.25 ± 0.2

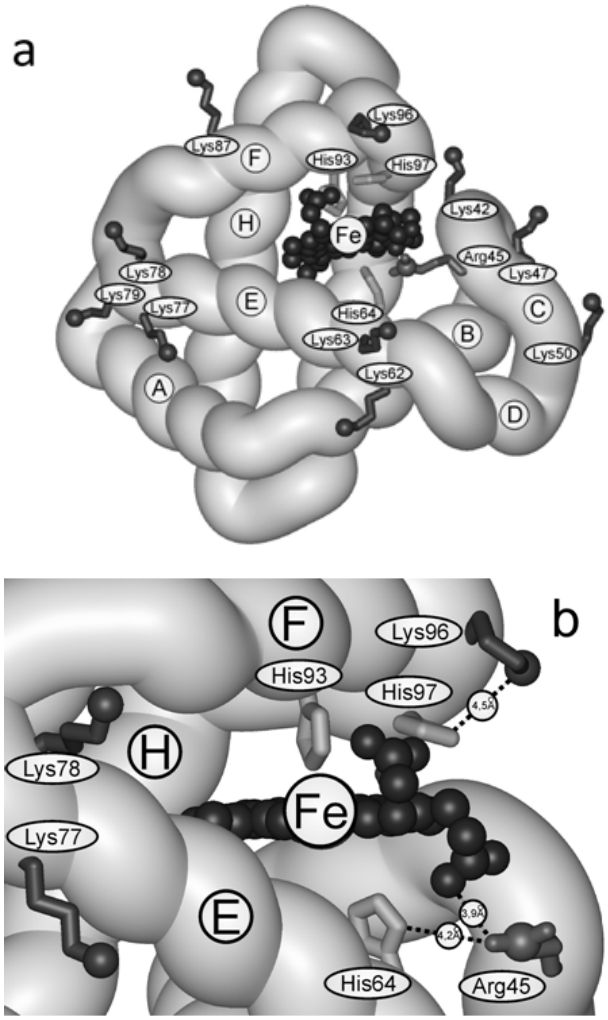


Fig. 10. *(a) The spatial structure of ligand-free sperm whale myoglobin and localization of invariant lysine and arginine residues in the environment of the heme cavity. The image was rendered using MOLMOL v2k2 and Corel Draw v. 11 software using the sperm whale myoglobin atomic coordinates from Protein Data Bank (PDB: 1VXA). (b) Fragment of the myoglobin structure near the heme. The proximal His93(F8) bound to Fe atom of the heme and the distal His64(E7), forming a hydrogen bond with O_2 ligand in the heme cavity are shown.*

2.5. Effect of Artificial and Natural Bilayer Phospholipid Membranes on the Conformation of Myoglobin and Its Affinity for Oxygen

In recent years it became evident that the conformation, stability and even functional properties of many water soluble proteins in cells significantly differ from their well studied properties in solution, as in cell compartments, they contact with various charged polymer structures, first of all, membranes. Due to electrostatic and hydrophobic interactions with a membrane surface, conformational state of proteins, which is very important for their functioning (transfer of different ligands, translocation through membranes, interaction with other proteins and protein complexes, oligomerization, etc.), can vary [41, 50, 51].

Myoglobin is known to readily associate with fatty acids, then it was assumed that it can serve as a mobile carrier of fatty acids to mitochondria [23-25]. Oxymyoglobin binds to fatty acids with a lower affinity than albumin, but like albumin, have the greatest affinity for unsaturated fatty acids and the lesser one for the saturated. Recent ^{1}H NMR-studies of palmitate interaction with myoglobin show that liganded ferric Mb(CN) and ferrous MbCO (the analog of physiologically active MbO_2) readily bind to the fatty acid, bur not ligand free Mb(2) [25], so the efficiency of binding is dependent on a liganded state of the protein. It is also shown that myoglobin can associate with artificial and natural bilayer phospholipid cell membranes and incorporate into artificial multilayer membranes [40, 52].

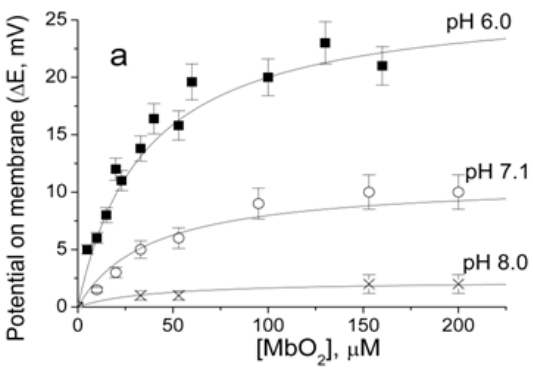
2.5.1. Interaction of Myoglobin with Neutral and Negatively Charged Artificial Bilayer Phospholipid Membranes [53]

Artificial bilayer phospholipid membranes (BLM) are excellent models of real biological structures and widely used for studying and interpreting the processes involving membranes of living cells. To simulate the interaction of myoglobin with phospholipids of the mitochondrial membrane, interaction of MbO_2 and metMb with BLMs from the neutral phospholipid, soybean phosphatidylcholine (lecithin), and from the negatively charged one, palmitoyl-2-oleyl-phosphatidylglycerol (POPG) was studied. Using potentiodynamic techniques known as the "capacity minimization" method [54-56], the potential difference between cis- and trans-sides of the membrane (ΔE, mV), which was generated by myoglobin adsorption on one of them, was measured. The ΔE dependence on MbO_2 and metMb concentration is similar to the Langmuir adsorption isotherm:

$$\Delta E = \Delta E_{max}\, c / (\alpha + c) \qquad (2)$$

where c is concentration of myoglobin, and $A = 1 / \alpha$ is the adsorption activity of the protein, corresponding to $\Delta E_{max} / 2$ [57].

The ΔE_{max} value is directly proportional to the surface charge density and related to the number of protein molecules adsorbed on BLM surfae and to the area they occupy [55]. In the case of MbO_2 (Fig. 11, a), $\Delta E_{max} = 27 \pm 2{,}5$ mV at pH 6, meaning that one elementary charge accounts for ~340 Å^2, which well correlates with the net charge of about +2 and the size of ~700 Å^2 of myoglobin molecule. Thus, the saturation potential ΔE_{max} corresponds to the state, when BLM surface is covered with monolayer of myoglobin.



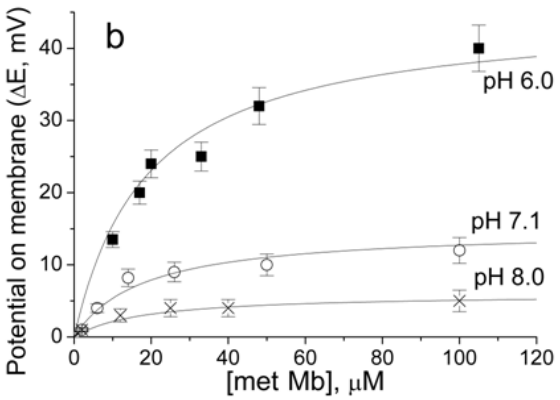


Fig. 11. *The dependence of surface potentials of the bilayer lecithin membrane on the concentration of sperm whale MbO_2 (a) and metMb (b) in the pH 6–8 range. Experimental conditions: KCl, 20 mM; Tris-maleate buffer, 15 mM. Solid curves are calculated Langmuir isotherms with: (a) $-\Delta E_{max}$ = 27 mV, α = 36 μM (at pH 6), ΔE_{max} = 11 mV, α = 38 μM (at pH 7.1) and ΔE_{max} 2.3 mV, α = 40 μM (at pH 8); (b) $-\Delta E_{max}$ = 45 mV, α = 19 μM (at pH 6), ΔE_{max} = 15 mV, α = 18 μM (at pH 7.1) and ΔE_{max} 6 mV, α = 18 μM (at pH 8).*

For lecithin BLM, ΔE dependences on MbO_2 and metMb concentration are shown at three pH values in the pH 6-8 range (Fig. 11, a, b). Saturation potentials (ΔE_{max}), answering to plateau on the curves, vary at different pH's because the net charge of adsorbed myoglobin changes in this pH range. The largest positive charge of the molecule is observed at pH 6 and the smallest one - at pH 8, the intermediate value is being at pH 7. In the case of metMb (Fig.11, b), the saturation potential recorded at the same pH is 1,5-2- fold higher than for MbO_2 (Fig.11, a), since metMb has an additional positive charge on the heme Fe atom.

The α parameter determined from $\Delta E/\ \Delta E_{max}$ dependence *versus c,* which characterizes the adsorption activity of MbO_2 and metMb with respect to lecithin BLM, is practically the same for all curves on Fig. 11,a and, respectively, Fig. 11,b, evidencing that the adsorption activity of MbO_2 and metMb does not depend on pH in the pH 6–8 range (Table 4). Hence, it does not depend on the net charge of myoglobin, changing in this pH interval. At the same time, the adsorption activity of metMb with respect to the neutral lecithin BLM is about two fold higher than that of MbO_2.

The adsorption of MbO_2 and metMb on the negatively charged BLM from POPG (POPG: lecithin: cholesterol ratio is 4:1:1) proceeds much more efficiently than on the lecithin BLM, as the saturation at pH 6 and 7.1 occurs at much lower myoglobin concentrations (Fig. 12,a,b). The experiment did not perform at pH 8 because of small ΔE_{max} values and large experimental errors therefore. The saturation potentials of MbO_2 and metMb on the negatively charged BLM from POPG are similar to ΔE_{max} values observed on the lecithin BLM at the same pH**,** since they are determined only by the net charge of the protein molecule. But the adsorption activity of MbO_2 and metMb relative to the negatively charged POPG membrane is about 15 and, respectively, 2,5 times higher, as compared to their activities relative to the neutral lecithin BLM (Table 4). Like to the lecithin BLM, the adsorption activity of both myoglobins with respect to the negatively charged BLM, does not depend on the net protein charge in the pH 6–8 range.

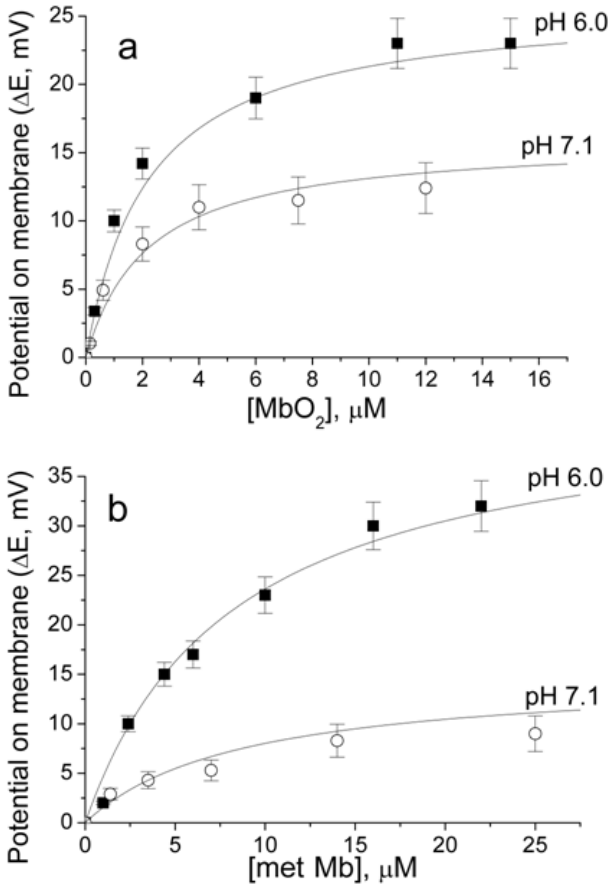


Fig. 12. *The dependence of surface potentials of the bilayer negatively charged phospholipid membrane from POPG (POPG: lecithin: cholesterol in the ratio 4:1:1) on the concentration of sperm whale MbO_2 (a) and metMb (b) in the pH 6–8 range. Experimental conditions as in Fig. 11. Solid curves are calculated Langmuir isotherms with: (a) – ΔE_{max} = 26 mV, α = 2.2 μM (at pH 6), ΔE_{max} = 15 mV, α = 2.2 μM (at pH 7.1); (b)– ΔE_{max} = 43 mV, α = 8.2 μM (at pH 6), ΔE_{max} = 15 mV, α = 8.7 μM (at pH 7.1).*

Table 4. *Parameters of the adsorption of sperm whale MbO_2 and metMb on a neutral lecithin bilayer membrane and negatively charged BLM of POPG (experimental conditions as in Fig.11 and 12).*

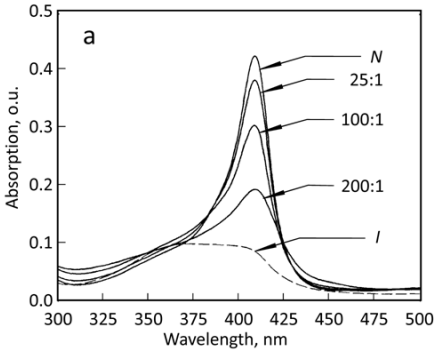
Sample	pH	Lecithin BLM			POPG BLM		
		ΔE_{max}, mV	α, μM	A =1/ α, x10^2 μM^{-1}	ΔE_{max}, mV	α, μM	A=1/ α, x10^2 μM^{-1}
MbO_2	6.0	27.0±2.5	36.0±3.5	2.8±0.3	26.0±2.5	2.2±0.5	45.5±2.0
	7.1	11.0±1.5	38.0±3.5	2.6±0.3	13.0±2.0	2.2±0.5	45.5±2.0
	8.0	2.5±0.5	40±4	2.5±0.3	-	-	-
метMb	6.0	45±3	19±2	5.3±0.5	43±3	8.2±1.0	12.2±1.0
	7.1	15.0±1.5	18±2	5.6±0.5	15±2	8.7±1.0	11.5±1.0
	8.0	6±1	18±2	5.6±0.5	-	-	-

Thus, MbO_2 and metMb readily bind to artificial phospholipid bilayer membranes, both to the neutral lecithin BLM and negatively charged BLM from POPG. The adsorption of both proteins is largely electrostatic in nature, since its efficiency substantially grows in using the negatively charged membrane. As the net charge of myoglobin molecule is shown to affect only ΔE recorded on the membrane, but not the adsorption activity of MbO_2 and metMb, local electrostatic interactions must take place between oppositely charged groups of membrane phospholipids and myoglobin, ionization state of which does not change in the pH 6–8 range. The fact that metMb associates with the lecithin and POPG membranes more efficiently than MbO_2 (in about two and four times, respectively) points again, in accordance with previous observations, to the involvement of the heme cavity region in the binding process. Invariant positively charged Lys and Arg residues in its surroundings (Fig. 10, a,b) might possibly participate in local electrostatic interactions with membrane phospholipids .

2.5.2. The Effect of Myoglobin Binding to BLM on the Protein Conformation

Binding of myoglobin to phospholipid membranes can markedly affect the native structure of the heme cavity and conformation of the molecule as a hole. To model effect of myoglobin interaction with negatively charged mitochondrial membranes, conformational changes of metMb in the presence of bilayer phospholipid liposomes (vesicles) from the negatively charged phospholipids, POPG and 1,2-dipalmitoyl-3-phosphatidylglycerol (DPPG), were studied in the pH 6–8 range at different molar phospholipids/metMb ratios using various physico-chemical methods [51, 58].

The metMb absorption spectrum in the visible spectral region allows to follow conformational changes in the nearest heme environments, which are induced by the presence of POPG liposomes (Fig. 13,a). At the molar POPG / metMb ratio, equal to 25 (pH 7.2), some small drop in the Soret band intensity is observed already, increasing when concentration of the liposomes rises. The maintenance of the spectrum shape evidences in favor of only minor conformational changes near the heme. According to our data (unpublished), liposomes from neutral lecithin affect the heme environment in metMb in much lesser extent, as the changes in the Soret band intensity (with preserving the spectrum shape) are observed only at lecithin / metMb ratio, equal to 200.



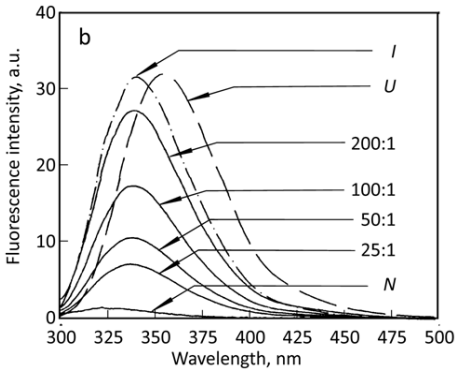


Fig. 13. *(a) Absorption spectra of sperm whale metMb in the Soret band in the presence of negatively charged phospholipid liposomes at different POPG / metMb ratios indicated at the curves. For comparison, spectra of metMb in native (N, pH 7.2) and intermediate (I, pH 3.6) states are given. 10 mM phosphate buffer; pH 7.2. (b) Changes in the tryptophan fluorescence of sperm whale metMb as dependent on the molar POPG / protein ratio (pH 7.2). For comparison, spectra of the native (N, pH 7.2), intermediate (I, pH 3.6) and fully unfolded (U, 6 M Guanidine hydrochloride) metMb forms are given. Excitation wavelength 293 nm, all measurements performed on a Shimadzu RF-5301 PC spectrofluorimeter using standard 1-cm path length quartz cuvette. The protein concentration is 0.05 mg/ml, 10 mM phosphate buffer.*

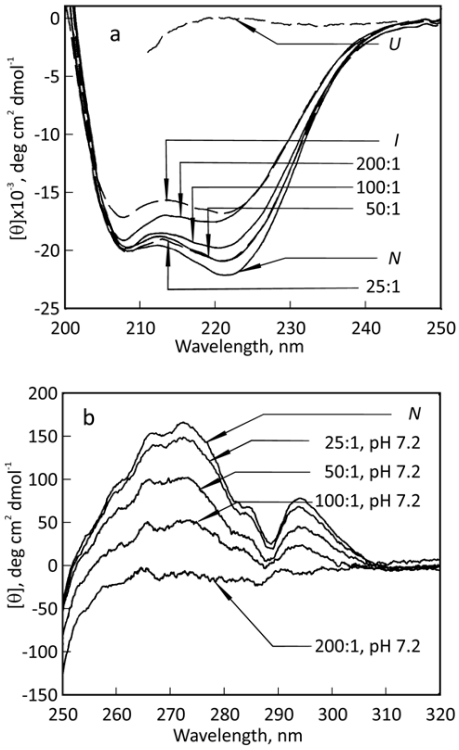


Fig. 14. *(a) Circular dichroism spectra of sperm whale metMb in the far UV spectral region at different POPG / metMb ratios indicated nearby the curves. For comparison, spectra of the native (N, pH 7.2), intermediate (I, pH 3.6) and fully unfolded (U, 6 M Guanidine hydrochloride) metMb forms are given. All CD spectra were registered on a JASCO J-600 spectropolarimeter using a 0.2 – mm path length cell. The protein concentration is 0.8 mg/ml., 10 mM phosphate buffer; pH 7.2. (b) Circular dichroism spectra of sperm whale metMb in the near UV spectral region at the different POPG / metMb ratios indicated nearby the curves. For comparison, the CD spectrum of native metMb (N, pH 7.2) is also given. CD spectra in near UV region were measured using a 10-mm path length cell. The protein concentration is 0.8 mg/ml, 10 mM phosphate buffer; pH 7.2.*

Two invariant tryptophans of A-helix in myoglobin, Trp7(A5) and Trp14(A12), allow to monitor conformational changes in metMb structure by fluorescence method, although tryptophanyl fluorescence of the holoprotein is strongly quenched by the heme group [44, 49]. At pH 7.2, some rising of the fluorescence with the shift of the spectral maximum to longer wavelengths is already observed at POPG / metMb ratio, equal to 25 (Fig. 13,b). With increasing concentration of POPG, further growth of the fluorescence intensity proceeds with maintaining the spectrum shape. The position of the metMb fluorescence maximum in the presence of POPG liposomes (338 nm), which is intermediate between that of native metMb (325 nm) and the fully unfolded form (355 nm), is usually attributed to partial exposure of tryptophan residues to water with preservation of a rougher compact protein structure. In the presence of liposomes, however, the influence of hydrophobic phospholipid membranes on the tryptophanyl fluorescence of bound metMb can not be excluded.

The circular dichroism (CD) of metMb in the far UV spectral region with two distinct minima at 208 and 220 nm is inherent for all α-helical proteins, providing information about their secondary structure. At 25-50-fold molar excess of POPG (pH 7.2), the CD spectrum is very close to the spectrum of the native protein in all respects (Fig. 14,a), thus indicating that the secondary structure of metMb is almost unchanged. Increasing concentration of POPG liposomes up to 100-fold excess of the phospholipid leads to further decrease in the molar ellipticity at 220 nm with maintenance of the spectrum shape, indicating further decrease in the number of metMb molecules with rigid tertiary structure. Marked changes in the CD spectrum shape of metMb at pH 7.2 take place only in the presence of 200-fold excess of POPG (Fig. 14,a). Nevertheless, all CD spectra of metMb in the presence of POPG liposomes are differ significantly from the spectrum of metMb in unfolded state, which means that the secondary helical structure of the protein remains quite pronounced, although it is not the same that of native metMb due to conformational changes in the tertiary structure.

The CD spectrum of metMb in the near UV spectral region evidences that in the presence of 25-fold molar excess of POPG at pH 7.2, tight packing of side groups in metMb is largely preserved (Fig. 14,b). The decrease in the ellipticity with increasing concentrations of POPG, while the spectrum shape is maintained, may be an indication to decreasing the number of metMb molecules with the rigid tertiary structure. The latter is completely lost at pH 7.2 only at POPG / metMb ratio, equal to 200, when amplitudes all characteristic bands of the CD spectrum became close to zero. It is important to underline that the interaction of myoglobin with negatively charged BLMs at physiological pHs leads first of all to conformational changes in the heme cavity of myoglobin, while essential disturbances of its secondary and tertiary structures do not occur. The conclusion is also supported by studies of the effect of the POPG and DPPG liposomes on a conformational stability of myoglobin, which were obtained using the high resolution 1H-NMR spectroscopy, scanning microcalorimetry, proteolysis, and many other methods [51, 58].

2.5.3. The Effect of Mitochondria and Artificial Phospholipid Membranes on the Rate of Sperm Whale Oxymyoglobin Autooxidation [59]

Conformational changes in the heme cavity induced by the interaction of myoglobin with phospholipid membranes must, in turn, affect its affinity for oxygen. Even small changes in the heme cavity structure of different monomeric globins having very similar spatial structures are known to lead to significant differences (tens or hundreds times) of their kinetic and equilibrium parameters of ligand binding [3, 27]. Large changes in the myoglobin affinity for oxygen (tens times) are achieved mainly by increasing the ligand dissociation rate, k_{-1}, whereas the association rate, k_1, little varies. Falling the affinity for O_2 with raising temperature also occurs primarily due to increasing the ligand dissociation rate [3, 27].

Alterations in MbO_2 / Mb(2) ratio in solution are difficult to detect under aerobic conditions, since the equilibrium in the reaction of myoglobin oxygenation is practically fully shifted to MbO_2 direction (Equation 1). However, studying the rate of MbO_2 autooxidation, i.e. spontaneous transition of MbO_2 to metMb under aerobic conditions (k_{ox}), allows to evaluate changes in the myoglobin affinity for O_2, which are induced by its interaction with phospholipid membranes, as a direct correlation between k_{ox} and the oxygen equilibrium dissociation constant (K_{dis}) has been found experimentally [60].

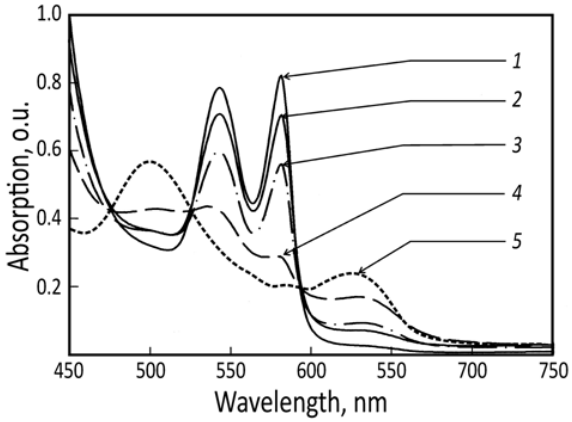


Fig. 15. *Changes in the absorption spectrum of sperm whale MbO_2 in the visible spectral region in the presence of the 25-fold molar excess of negatively charged POPG liposomes. 10 mM Tris-HCl buffer, pH 7.2 (37^{0}C). 1 – the original spectrum of native MbO_2 without liposomes (the control); 2 – the same after 3.5 hours of incubation at 37^{0}C; 3 – MbO_2 spectrum in the presence of 25-fold molar excess of POPG (40 min after mixing); 4 – the same after 6 hours; 5 – metMb spectrum after adding a 100-fold molar excess of POPG liposomes. Bilayer phospholipid vesicles (liposomes) were prepared using a standard procedure of sonication.*

The effect of negatively charged POPG liposomes on MbO_2 autooxidation at different phospholipid / MbO_2 ratios at 37^{0}C (pH 7.2) is shown on Fig. 15. Without liposomes, the absorption spectrum of MbO_2 in the visible spectral region with two distinct intense peaks at 543 and 581 nm (Curve 1) only slightly changes after 3,5 hours (Curve 2), but in the presence of 25-fold excess of POPG, a significant part of

MbO_2 autooxidizes to metMb already in 40 min (Curve 3), while after six hours, MbO_2 almost completely turns into metMb, characterizing by two much less intense peaks at 505 and 634 (Curve 4). Thus, in the presence of 25-fold molar excess of POPG (pH 7.2, 37^0C), k_{ox} is ~20-fold increased as compared to that without liposomes. In the presence of 100-fold molar excess of POPG, all MbO_2 immediately transfers to metMb (Fig. 15, curve 5).

The autooxidation process slows down considerably at room temperature, which makes possible to compare the effect of neutral and negatively charged liposomes on k_{ox} at various phospholipid / MbO_2 ratios (Fig. 16). Compared to the control (Curve 1), k_{ox} is accelerated 8 and 25 times in the presence of 50- and 100-fold excess of lecithin, respectively (Curves 2 and 4). In the presence of 25-and 100-fold molar excess of POPG, it rises much more, 22 and 174 times, respectively (Curves 3 and 5). So, MbO_2 autooxidazes almost 3 times faster in the presence of 25-fold molar excess of POPG, than of 50-fold excess of lecithin, and roughly with the rate corresponding to that for 100-fold excess of lecithin. In turn, at 100-fold excess of POPG liposomes, k_{ox} is 7 times higher than that for those from lecithin.

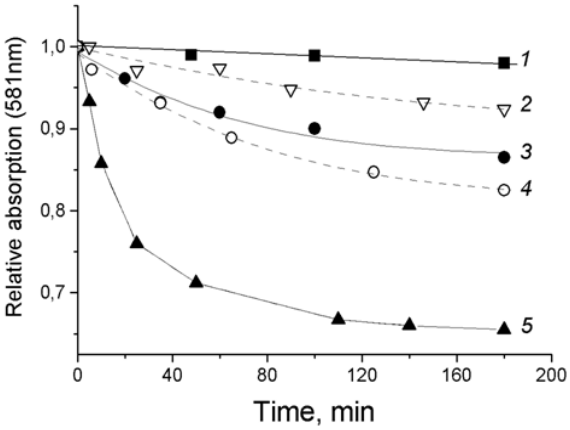


Fig. 16. *Kinetics of sperm whale MbO_2 autooxidation without liposomes and in the presence of neutral and negatively charged liposomes at different phospholipid / protein ratios. 10 mM Tris-HCl buffer, pH 7.2, 22^0C. 1 – MbO_2 without liposomes (the control), 2 and 4 – MbO_2 in the presence of 50- and 100-fold molar excess of lecithin, respectively, 3 and 5 – MbO_2 in the presence of 25- and 100-fold molar excess of POPG, respectively. MbO_2 concentrations 0.11 mM.*

Similarly, the rate of MbO_2 autooxidation increases in the presence of no respiring mitochondria inhibited by antimicyne A (Fig. 17). Compared to the control (Curve 1), k_{ox} rises about 10- and 20-fold proportionally to the concentration of mitochondria in the suspension (Curves 2 and 3). Since mitochondria can be a source of reactive oxygen species which can rapidly oxidize MbO_2, the effect of superoxide dismutase (SOD) on MbO_2 autooxidation rate in the presence of mitochondria inhibited by antimycin A was studied. It was found that the recorded k_{ox} almost unchanged when adding SOD to the incubation medium at low (curve 5) and high (curve 6) ionic strengths (the k_{ox} changes are within the experimental error).

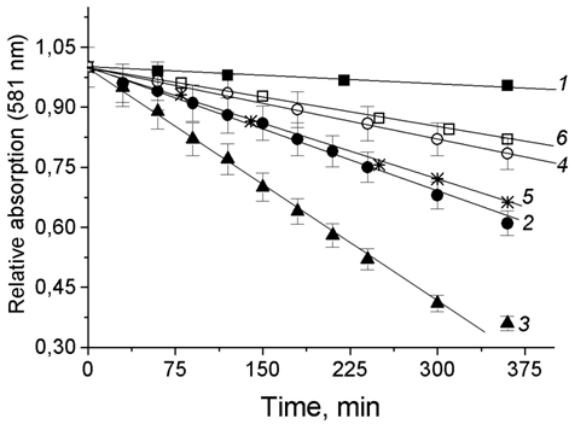


Fig. 17. *Kinetics of sperm whale MbO_2 autooxidation in the presence of mitochondria inhibited by antimycin A. Effect of ionic strength and superoxide dismutase (SOD) on the rate of the process. All measurements were performed in cuvette with optical path length of 0.5 cm using the spectrophotometer compartment for turbid samples to reduce the light-scattering effect. The MbO_2 concentration is 0.11 mM. Incubation medium without succinate, 10 mM Tris-HCl buffer, sucrose, 250 mM; EGTA, 0.5 mM; KH_2PO_4, 5 mM, 20 μM antimycin A, pH 7.2, 22^0C (low ionic strength) and the same plus 100 mM KCl (high ionic strength). 1 – MbO_2 without mitochondria (the control); 2 – 3 –concentration of mitochondria is 1 and 2 mg/ml mitochondrial protein, respectively (low ionic strength); 4 –1 mg/ml of mitochondrial protein (high ionic strength); 5 – 1 mg/ml of mitochondrial protein (low ionic strength) plus 3 mM SOD per the heme, 6 – the same plus 100 mM KCl.*

The O_2-complex of the ferrous heme in solution is not stable because of very rapid and irreversible autooxidation to the ferric heme, whereas native sperm whale MbO_2 autooxidazes very slowly. The process takes several days at pH 7.2 and room temperature, and markedly accelerated with decreasing pH and increasing temperature [60, 61]. The high stability of this complex in MbO_2 and HbO_2 is shown to be due to hydrophobic environments of the heme and H-bond between the liganded O_2 and the distal His64(E7) in the heme cavity (Fig. 18, a,b). The autooxidation of MbO_2 is initiated by breaking this hydrogen bond with the subsequent dissociation of superoxide (the rate limiting step of the reaction) [61, 62]. Therefore, the hydrogen bonding of the neutral His64(E7) with the liganded O_2 molecule plays a particularly important role in the inhibition of MbO_2 autooxidation, as it hinders both dissociation of the bound oxygen and protonation of the FeO_2-complex, which is responsible for k_{ox} dependence on pH. Replacement of the distal His64(E7) by nonpolar amino acid residues, incapable to H-binding with O_2, results in 100-800-fold increasing of MbO_2 autooxidation rate [60].

$$Mb(2)O_2 + O_2 + 2H^+ \xrightarrow{k_{ox}} Mb(3)H_2O + O_2 \qquad (3)$$

We could see that the interaction of MbO_2 with mitochondrial and artificial phospholipid membranes leads to significant increasing its autooxidation rate, the effect of negatively charged membranes on k_{ox} being much greater than of neutral ones. It is in accord with other above observations and points to the important role of electrostatics in this interaction. The direct correlation between k_{ox} and K_{dis} strongly suggests that both processes are governed by the same structural features of myoglobin, first of all, the heme

cavity conformation. Together with the heme-protein hydrophobic interactions and the H – bond between distal His64(E7) and liganded O_2, electrostatic interactions also make a significant contribution to stabilization of the native structure of the heme cavity. They involve interactions between the heme 6-propionate, Arg45(CD3), Asp60(E3) and His64, as well as the "salt bridge" between the heme-1-propionate and His97(FG2), stabilizing His64(E7) position, in which it is capable to donate hydrogen to the liganded O_2 (Fig. 18, a,b).

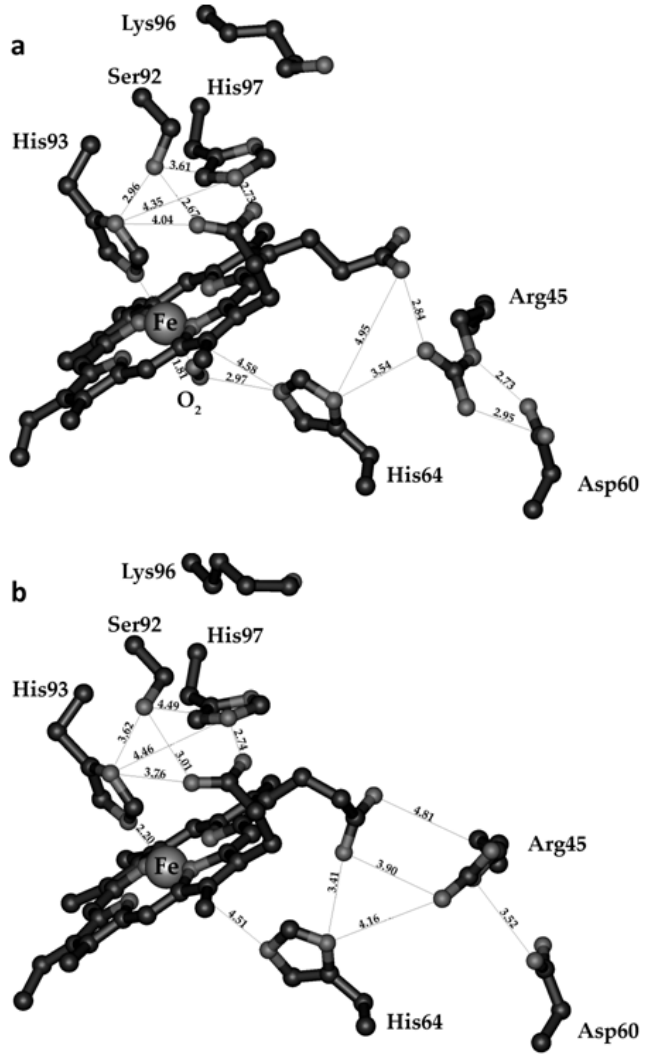


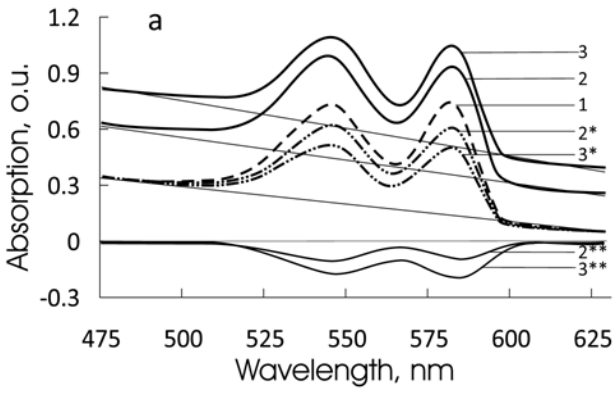
Fig. 18. *The structure of the distal part of the heme pocket in the oxy- (a) and deoxy- (b) sperm whale myoglobin. Atomic coordinates of myoglobin spatial structures are taken from the database PDB 1A6M and 1VXA and visualized using the Mol Mol program. The distances between atoms are given in angstroms*

If, using site-specific mutagenesis, Lys45 in pig myoglobin (equivalent of Arg45 in sperm whale) is replaced by His which is also interacts with the heme 6-propionate, Asp60 and His64, although less efficiently than Lys or Arg in this position, the autooxidation rate increases 3 times, while replacement of Lys45 by small and uncharged Ser – 7 times [60]. The natural homologous Arg45–Lys45 mutation in sperm whale and pig myoglobins only very slightly affects K_{dis} and k_{ox}, and can be regarded as a neutral with respect to the autooxidation. The dramatic nearly 20-fold increase of k_{ox} is observed when Lys45 was replaced by Glu. It is obvious that the loss of the positive charge at position 45, or even adding therein the negative charge disturbs the native configuration of this area, possibly increasing the accessibility of the distal pocket to solvent. Electrostatic interactions MbO_2 with artificial and natural phospholipid membranes may well be the reason for similar disturbances in the heme cavity conformation, which leads to the increased autooxidation rate of myoglobin under aerobic conditions and, in turn, points to decreased affinity of the protein for the ligand.

2.6. The Effect of Phospholipid Membranes on the Oxy- / Deoxymyoglobin Equilibrium

It has long been noted that oxygen affinity of myoglobin is 4-5 times lower in muscle cells than in solution at $37^{O}C$, although no compounds are known yet that are capable to significantly change p_{50} of myoglobin, like diphospho glycerate, protons and CO_2 affecting p_{50} of hemoglobin [3, 26, 27]. Due to very high myoglobin affinity for O_2 (p_{50} is 0,6–0,7 mm Hg at $25^{0}C$), some changes of MbO_2 / Mb(2) ratio, which are caused by myoglobin interaction with phospholipid membranes, can be registered only under anaerobic conditions, when oxygen concentration in solution is close to zero.

In our experiments, therefore, oxygen was removed from MbO_2 solution, as earlier, in the polarographic cell using respiring mitochondria put in the tightly closed dialysis bag (as noted above, no MbO_2 deoxygenation is observed, Fig. 2, a). Then, at p_{02} in the cell near zero, nonrespiring mitochondria inhibited by antimycin A or lecithin liposomes were added [62-64]. In the presence of no respiring mitochondria, a decrease in the absorbance at 542 and 581 nm, which is intrinsic for MbO_2 spectrum, is observed, while the absorbance at 560 nm, in the spectral maximum of Mb(2) increases (Fig. 19, a). The shift of MbO_2 / Mb(2) equilibrium towards the ligandfree Mb(2) rises proportionally to concentration of mitochondria, which is clearly seen from the difference spectra (Fig. 19, a, curves 2** and 3**). The same effect is observed in the presence of lecithin liposomes at 50-fold molar excess of lecithin (Fig. 19, b). It is evident that the equilibrium O_2 dissociation constant (K_{dis}) rises in both cases due to interaction of the protein with phospholipid membranes.



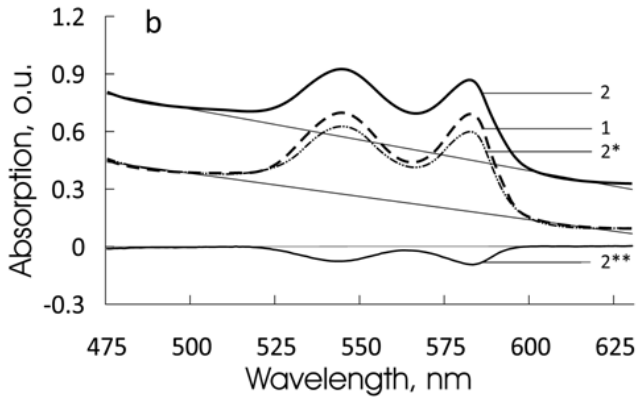


Fig. 19. *The shift of the oxy- / deoxymyoglobin equilibrium in the presence of mitochondria inhibited by antimycin A (a) and lecithin liposomes (b). All measurements were performed in cuvette with optical path length of 1 cm using the Specord UV VIS spectrophotometer and compartment for turbid samples to reduce the light-scattering effect. The concentration of sperm whale MbO_2 is 0.05 mM. a) 1 – MbO_2 spectrum without mitochondria (the control), 2 and 3 – the same in the presence of mitochondria, 1 and 2 mg/ml of mitochondrial protein, correspondently, 2* and 3* – the same with deduction of the light scattering, 2** and 3** — the difference (2* minus 1 and 3* minus 1) spectra. Incubation medium without succinate (low ionic strength) and experimental conditions as in Fig.17, antimycin A, 20 μM, pH 7.4, 22^{0}C. b) 1 – MbO_2 without liposomes (the control), 2 – MbO_2 in the presence of 50-fold molar excess of lecithin, 2*– the same with deduction of the light scattering, 2** – the difference (2* minus 1) spectrum. 10 mM Tris-HCl buffer, pH 7.4, 22^{0}C. Bilayer phospholipid vesicles (liposomes) were prepared using the standard procedure of sonication.*

3. Conclusion

Thus, detachment of oxygen from MbO_2 at physiological p_{02} values occurs only upon interaction of myoglobin with the mitochondrial membrane, which results in a decreased myoglobin affinity for oxygen due to conformational changes in the protein heme cavity. Hence, in the presence of respiring mitochondria, at least two kinds of myoglobin molecules should be observed: some are free, having a high affinity for O_2, and respectively, a very low p_{50} value, and the others that are associated with mitochondria and have lower affinities and higher p_{50} values. The situation should be described as follows:

$$\mathrm{Mb(2) + O_2} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{MbO_2 + MC} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{[MbO_2 \bullet MC]} \underset{k_1'}{\overset{k_{-1}'}{\rightleftarrows}} \mathrm{Mb(2) + MC \bullet O_2} \quad (4)$$

where k_1 and k_{-1} are the O_2 binding and dissociation constants of myoglobin in solution (Equation 1), k_2 and k_{-2} – constants of formation and decay of myoglobin - mitochondrial complex, respectively, and k_1' and k_{-1}' are those of myoglobin bound to mitochondria. The difference between the k_1' and k_{-1}' constants and corresponding constants without superscripts is evidently due to some changes in the heme cavity conformation because of interaction of myoglobin with the mitochondrial membrane.

On the basis of obtained results and analysis of myoglobin structure, it can be assumed that upon complexion of myoglobin with mitochondria, local electrostatic interactions must take place between phospholipid polar groups of a bilayer membrane (the heads) and oppositely charged groups around the heme cavity of myoglobin, the ionization state of which does not change in the pH 6–8 range. The most likely candidates to interact with the anionic groups of phospholipids are invariant arginine and lysine residues in the environment of the heme cavity [Fig. 10, 18]. Long ago many investigators drew attention that in all myoglobins, a flat ground of seven invariant charged residues in the bend between the C and D helices is located near the heme and could serve as an anchor for the binding to mitochondria and some other cellular structures [3, 27, 65]. Computer calculations of dipole moments of different mammalian myoglobins (Sivozhelezov V.S., unpublished data) show that the positive end of vector of the dipole moment (200 D) in all the cases is situated in the area 88–91 residues of F helix, while the proximal His93(F8) associated with the Fe atom is located from the opposite side.

It should be noted that for effective O_2 transfer from cytoplasm, myoglobin should not form stable complexes with the mitochondrial membrane, so that the exchange between the two types of MbO_2 molecules, free and membrane-bound ones, has to be rather fast, to proceed, for example, at the rate of rotational diffusion of the protein just to alter an orientation of the heme cavity relative to a membrane surface. Otherwise, myoglobin could not effectively accelerate O_2 delivery to mitochondria under oxygen deficit in the cell. The K_m values determined for the MbO_2 binding to mitochondria using 1,8-ANS and M 540 fluorescent probes (about 10^4 M^{-1} at I = 0,15), and the lifetime of the complex (tens ns) correspond well to the middle (not high) myoglobin affinity for the mitochondrial membrane. The rate of rotational diffusion of myoglobin in muscle cells is found to be only 1,5 times lower, than in the dilute water solution [10, 12-14], which is optimal for the effective functioning of myoglobin as the O_2 transporter.

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References

[1] Hill, R.L. Oxygen affinity of muscle haemoglobin. Nature, 1933; 132: 897-898

[2] Millikan, G.A. Muscle hemoglobin. Physiol. Rev., 1939; 19: 503-523.

[3] Starodub, R.F., Korobov, V.N., and Nazarenko, V.I. Myoglobin: structure, properties, synthesis and biological role. Ed. by «Naukova Dumka» (Kiev), 1992, p.p. 1-281.

[4] Wittenberg J.B. Myoglobin facilitated oxygen diffusion and the role of myoglobin in oxygen entry into muscle. Physiol. Rev. 1970; 50: 559-636.

[5] Wittenberg, B.A., Wittenberg, J.B., and Caldwell, P.R.B. Role of myoglobin in the oxygen supply to red skeletal muscle. J. Biol. Chem. 1975; 250: 9038-9043.

[6] Wittenberg, J.B., and Wittenberg, B.A. Facilitated diffusion by oxygen carriers. In: Oxygen and Living Processes. Ed. Gilbert, D.L., Springer–Verlag, New York, 1981, pp.177-199.

[7] Wittenberg, B.A., and Wittenberg, J.B. Transport of oxygen in muscle. Ann. Rev. Physiol. 1989; 51: 857-878.

[8] Hemingsen, E. Enchancement of oxygen transport by myoglobin. Comp. Biochem. Physiol. 1963; 10: 239-244.

[9] Fletcher J.E. On facilitated oxygen diffusion in muscle tissues. Biophys. J. 1980; 29: 437-458.

[10] Jelicks I.A., and Wittenberg B. A. 1H nuclear magnetic resonance studies of sarcoplasmic oxygenation in the red cell-perfused rat heart. Biophys.J. 1995; 68: 2129-2136.

[11] Jurgens K.D., Papadopoulos S., Peters T., and Gross G. Myoglobin: just an oxygen store or also an oxygen transporter? News Physiol. Sci. 2000; 15: 269-274.

[12] Whiteley J.P., Gavaghan D.J., and Hahn C.E.W. Mathematical modeling of oxygen transport to tissue. J. Math. Biol. 2002; 44**:** 503-522.

[13] Garry D.J., Ordway G.A., Lorenz J.A., Radford N.B., Chin E.R., Grange R.W., Bassel-Duby Rh., and Williams R.S. Mice without myoglobin. Nature 1998; 395**:** 905-908.

[14] Vynogradov A.D. What for myoglobin is needed? Biochemistry (Moscow) 1999; 64: 709-710.

[15] Skulatchev, V.P. Comments to the paper by Vynogradov A.D. "What for myoglobin is needed?" Biochemistry (Moscow) 1999; 64: 711-712.

[16] Wittenberg J.B., and Wittenberg B.A. Myoglobin function reassessed. J. Experim. Biol. 2003; 206: 2011-2020.

[17] Gros, G., Wittenberg B.A., and Jue, Th. Myoglobin's old and new clothes: from molecular structure to function in living cells (review). J. Experim. Biol. 2010; 213**:** 2713-2725.

[18] Flogel, U., Merx, M. W., Godecke, A., Decking, U. K., and Schrader, J. Myoglobin: a scavenger of bioactive NO. Proc. Natl. Acad. Sci. USA. 2001; 98: 735-740.

[19] Creutzer, U., and Jue, Th. Investigation of bioactive NO-scavenging role of myoglobin in myocardium. Eur. J. Physiol. 2006; 452: 36-42.

[20] Rassaf, T., Flogel, U., Drexhage, C., Hendgen-Cotta, U., Kelm, M., and Schrader, J. Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetic and function. Circ. Res. 2007; 100: 1749-1754.

[21] Flogel, U., Godecke, A., Clotz, L.-O., and Schrader, J.. Role of myoglobin in the antioxidant defense of the heart. FASEB J. 2004; 18: 1156-1158.

[22] Gloster, J., and Harris, P. Fatty acid binding to cytoplasmic proteins of myocardium and red and white skeletal muscle in the rat. A possible new role for myoglobin. Biochem. Biophys. Res. Communs. 1977; 74: 506-513.

[23] Gotz, F.M., Hertel, M., and Groschel-Stewart, U. Fatty acid binding of myoglobin depends on its oxygenation. Biol. Chem. Hoppe-Seyler, 1994; 375: 387-392.

[24] Shin, L., Chung, Y., Stiram, R. and Jue, Th. Palmitate interaction with physiological states of myoglobin. Biochimica et Byophysica Acta 2014; 1840: 656-666.

[25] Tilakaratne, H.K., Hunter, S.K., and Rodgers, V.G.J. Mathematical modeling of myoglobin facilitated transport of oxygen in devices containing myoglobin-expressing cells. Math. Biosciences 2002; 176: 253-267.

[26] Antonini E., Brunori M. Hemoglobin and myoglobin in their reactions with ligands. Ed. Amsterdam–London: Front Biol., 1971, pp. 1-415.

[27] Sharonov, Yu.A., and Sharonova N.A. Structure and functions of hemoglobin. Mol. Biol. (Moscow) 1975; 9: 145-172.

[28] Chang, Y., and Jue, Th. Regulation of respiration in myocardium in the transient and stady state. Am. J. Physiol. Heart Circ. Physiol. 1999; 277: H1410-H1417.

[29] Arai, A.E., Kasserra, C.E., Territop, P.R., Gandjibakhche, A.H. & Balaban, R.S. Myocardial oxygenation in vivo: optical spectroscopy cytoplasmic myoglobin and mitochondrial cytochromes. Am J. Physiol. 1999; 277: H683- H697.

[30] Postnikova G.B. and Tselikova S.V. Myoglobin and mitochondria: kinetics of oxymyoglobin deoxygenation in a mitochondrial suspension. Biophysics (Moscow) 2005; 50: 284-291.

[31] Shuruta S.A., Amerkhanov Z.G., Postnikova G.B. Effect of mitochondria on the redox reaction between oxymyoglobin and ferricytochrome c. Biophysics (Moscow) 1999; 44: 1023-1026.

[32] Enoki Y., Matzumura K., Ohga Y., Kohzuki H., and Hattori M. Oxygen affinities (p_{50}) of myoglobins from four vertebrate species (Canis familiaris, Rattus norvegicus, Mus musculus and Gallus domesticus) as determined by a kinetic an equilibrium method. Comp. Biochem. Physiol. 1995; 110B: 193-199.

[33] Papadopoulos, S., Endeward, V., Revesz-Walker, B., Jurgens, K.D., and Gross, G. Radial and longitudinal diffusion of myoglobin in single living heart and skeletal muscle cells. Proc. Natl. Acad. Sci. USA 2001; 98: 5904-5909.

[34] Lin, P.-Ch., Kreutzer, U., and Jue, Th. (2001). Myoglobin translational diffusion in rat myocardium and its implication on intracellular oxygen transport. J. Physiol. 2001; 578.2: 595-603.

[35] Postnikova G.B., Tselikova S.V., Shekhovtsova E.A. Myoglobin and mitochondria: oxymyoglobin interacts with mitochondrial membrane during deoxygenation. Biochemistry (Moscow) 2009; 74: 1211-1218.

[36] Wittenberg, J.B., and Wittenberg, B.A. Myoglobin-enhanced oxygen delivery to isolated cardiac mitochondria. J. Experim. Biol. 2007; 210: 2082-2090.

[37] Cannon, J.B., Kuo, F.S., Pasternack, R.F., Wong N.M., and Muller-Eberhard, U. Kinetics of the interaction of hemin liposomes with heme binding proteins. Biochemistry 1984; 23: 3715-3721.

[38] Tang, J., Faustman, C., Hoagland, Th.A., Mancini, R.A., Seyfert, M., and Hunt, M.C. Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. J. Agric. Food Chem. 2005; 53: 1223-1230.

[39] Jensen, L.J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Doerks, T., Julien, P., Roth, A., Simonovic, M., Bork, P., and von Mering, C. Protein data bank. Nucleic Acids Res. 2009; 37**:** D412-D416.

[40] Ramandeep, Hwang, K.W., Raje Manoj, Kim, K.-J., Stark, B.C., Dikshit, K.L., and Webster, D.A. Vitreoscilla hemoglobin. Intracellular localization and binding to membranes. J. Biol. Chem. 2001; 276: 24781-24789.

[41] Bychkova, V.E., and Ptitsin, O.B. The molten globule in vitro and in vivo. Chemtracts – Biochem. and Mol. Biol., 1993; 4: 133-163.

[42] Postnikova G.B., Shekhovtsova E.A. Fluorescence studies on the interaction of myoglobin with mitochondria. Biochemistry (Moscow) 2012; 77: 280-287.

[43] Vladimirov,Yu.A., Dobretsov, G.E. Fluorescent probes in biological membranes studies. Ed. «Nauka», Moscow, 1980, 1-320.

[44] Postnikova G.B. Fluorescence study of conformational transitions in the structure of myoglobin (review). Biochemistry (Moscow) 1999; 64: 267-286.

[45] Robertson D.E., Rottenberg H. Membrane potential and surface potential in mitochondria. Fluorescence and binding of 1-anilinonaphthalene-8-sulfonate. J. Biol. Chem. 1983; 258: 11039-11048.

[46] Verkman, A.S. Mechanism and kinetics of merocyanine 540 binding to phospholipids membranes. Biochemistry 1987; 26: 4050-4056.

[47] Lelkes, P.I., and Miller, I.R. Perturbations of membrane structure by optical probes: I. Location and structural sensitivity of merocyanine 540 bound to phospholipids membranes. J. Membrane Biol. 1980; 52: 1-15.

[48] Smith, J.C., Graves, J.M., and Williamson, M. The interaction of the potential – sensitive molecular probe merocyanine 540 with phosphorylating beef heart submitochondrial particles under equilibrium and time – resolved conditions. Archives. Biochem. Byophys. 1984; 231: 430-453.

[49] Postnikova G.B., Komarov Yu. E., and Yumakova E.M Fluorescence study of the conformational properties of myoglobin structure. 2. pH- and ligand-induced conformational changes in ferric and ferrous myoglobins. Eur. J. Biochem. 1991; 198: 233-239.

[50] Bychkova, V.E. About of the functional role of non active proteins. Uspekhi biologycheskoi khimii (Moscow) 1997; 37: (1), 49-99.

[51] Basova, L.V. Studies of the influence of membrane surface on the conformational state of globular proteins (PhD. Diss.), Pushchino, Moscow region, 2004, p.p. 1-126.

[52] Nassar, A-E. F., Zhang, Zh., Chynwat, V., Frank, H.A., Russling, J.F., and Suga, K. Orientation of myoglobin in cast multibilayer membranes of amphiphilic molecules. J. Phys. Chem. 1995; 99: 11013-11017.

[53] Grigorijev P.A., Postnikova G.B., and Shekhovtsova E.A. Study of the interaction of myoglobin with lipid bilayer membranes by potentiodynamic method. Biophysics (Moscow) 2012; 57: 55-60.

[54] Sokolov V.S. and Kuzmin V.G. Study of surface potential difference in bilayer membranes according to the second harmonic response of capacitance current. Biofizika (Moscow) 1980; 25: 170-172.

[55] McLaughlin, S.G.A, Szabo, G., and Eizenman, G., Divalent ions and the surface potential of charged phospholipid membranes. J. Gen. Physiol. 1971; 58: 667–687.

[56] Suznjevic, D., Grigorijev P.A., and Vucelic D. Study of LBM interactions with some surface - active substances by the method of capacity minimization. Bioelectrochemistry and Bioenergetics 1994; 34: 19-24.

[57] Shchukin,E. D., Pertsov, A.B., and Amelina, E.A. Colloid chemistry. Ed. «Vysschaya schkola» (Moscow) 2007, p.p. 1-418.

[58] Basova, L.V., Tiktopulo, E.I., Klenin, S.I., and Bychkova, V.E. Negatively charged phospholipid vesicles affect tertiary structure of holomyoglobin at neutral pH. Biophis. J. 2004; 86: 617a.

[59] Postnikova G.B. & Shekhovtsova E.A. The effect of artificial and natural phospholipid membranes on the rate of sperm whale oxymyoglobin autooxidation. Biochemistry (Moscow) 2013; 78: 357-363.

[60] Brantley, R.E., Smerdon, S.J., Wilkinson, A.J., Singleton, E.W., and Olson, J.S. The mechanism of autooxidation of myoglobin. J. Biol. Chem. 1993; 268; 6995-7010.

[61] Shikama, K. The molecular mechanism of autoxidation for myoglobin and hemoglobin: a venerable uzzle. Chem. Review 1998; 98: 1357-1373.

[62] Postnikova, G.B., Shekhovtsova, E.A. Myoglobin and mitochondria: How does the “Oxygen store” work? J. Phys. Chem. Biophys. 2013; 3: 126.

[63] Postnikova, G.B., Shekhovtsova, E.A. Myoglobin acts as the oxygen carrier to mitochondria. Physiology Science 2014; 1: 12–16.

[64] Postnikova, G.B., Shekhovtsova, E.A. The interaction of myoglobin with neutral and negatively charged artificial bilayer phospholipid membranes. Their effect on conformation of myoglobin and its affinity for oxygen. Physiology Science 2014; 1: 1–11.

[65] Romero-Herrera, A.E., Lehmann, H., Joysey, K.A., Friday A.E. On the evolution of myoglobin. Philos. Trans. R Soc. London B. Biol. Sci. 1978; 283 (1); 61-75.