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Fluorescence Studies on the Interaction of Myoglobin with Mitochondria

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Abstract—To determine the nature and characteristic parameters of the myoglobin–mitochondrion interaction during oxymyoglobin (MbO_2) deoxygenation in the cell, we studied the quenching of the intrinsic mitochondrial flavin and tryptophan fluorescence by different liganded myoglobins in the pH range of 6-8, as well as the quenching of the fluorescence of the membrane probes 1,8-ANS and merocyanine 540 (M 540) embedded into the mitochondrial membrane. Physiologically active MbO_2 and oxidized metmyoglobin (metMb), which are unable to bind oxygen, were used as the quenchers. The absence of quenching of flavin and tryptophan fluorescence implies that myoglobin does not form quenching complexes with either electron transport chain proteins of the inner mitochondrial membrane or with outer membrane proteins. We found, however, that MbO_2 and metMb effectively quench 1,8-ANS and M 540 fluorescence in the pH range of 6-8. Characteristic parameters of 1,8-ANS and M 540 fluorescence quenching by the myoglobins (extent of quenching and quencher binding constant, K_m) are very similar, indicating that both probes are localized in phospholipid sites of the mitochondrial membrane, and myoglobin is complexed with these sites. The dependence of K_m on ionic strength proves the important role of coulombic interactions in the formation of the quenching complex. Since the overall charge of myoglobin is shown not to influence the K_m values, the ionic strength dependence must be due to local electrostatic interactions in which polar groups of some part of the myoglobin molecule participate. The most likely candidates to interact with anionic groups of mitochondrial phospholipids are invariant lysine and arginine residues in the environment of the myoglobin heme cavity, which do not change their ionization state in the pH range investigated.

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Key words: myoglobin, mitochondria, membrane, fluorescence

We have found a novel mechanism of oxymyoglobin (MbO_2) function in the cell, which, unlike commonly accepted mechanisms of "oxygen depot" and "facilitated diffusion", includes the interaction of MbO_2 with mitochondria during deoxygenation [1, 2]. The result of this interaction is a change in conformation of the heme cavity of myoglobin and, as a consequence, decrease in its affinity to a ligand, facilitating O_2 release at physiological oxygen concentrations (p_{O_2}) in the cell. Our data on the competitive effect on the MbO_2 deoxygenation rate in the presence of mitochondria or of other proteins (a structural homolog of the holoprotein apomyoglobin, negatively charged lactalbumin and BSA, and positively charged lysozyme) suggest the absence of myoglobin-specific sites on the mitochondrial membranes, but indicate that, during deoxygenation, MbO_2 most likely interacts with phospholipid domains of the outer membrane, and electrostatic forces play an important role in this interaction [2].

In this work, in order to study in more detail the nature of the interaction of myoglobin with mitochondria and to determine its characteristic parameters, we used the fluorescence method. Since the heme group is an efficient fluorescence quencher for various donors, we studied the quenching of intrinsic tryptophan and flavin fluorescence of mitochondria and fluorescence of membrane probes 1,8-ANS and merocyanine 540 (M 540) by differently liganded myoglobins at pH 6-8 and ionic strength 10-150 mM. Both membrane probes are widely used in studies of natural and artificial membranes. While 1,8-

Abbreviations: 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; K_m, constant of quencher binding with mitochondria; M 540, 5-[(3-γ-sulfopropyl-2(3H)-benzoxazolylidene)-2-butenylidene]-1,3-dibuthyl-2-thiobarbituric acid; MbO_2, oxymyoglobin; metMb, metmyoglobin; P, fluorescence polarization degree; p_{O_2}, oxygen partial pressure; q, fluorescence quantum yield.

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ANS can bind to both lipids and hydrophobic protein domains, M 540 is specific to phospholipids [3]. The quenchers used were physiologically active MbO_2 capable of oxygen release and inactive oxidized metMb unable to bind oxygen.

It was found that neither myoglobin quenched the intrinsic flavin and tryptophan fluorescence of mitochondrial suspension at pH 6-8. The absence of quenching is indicative of the absence of complex formation between myoglobin and electron transport chain proteins of the inner mitochondrial membrane or proteins of the outer membrane as well. In contrast, efficient quenching the 1,8-ANS and M 540 fluorescence by MbO_2 and metMb was observed. Parameters of quenching by myoglobin (quenching degree and quencher binding constant) are very similar for the two probes. This suggests that both 1,8-ANS and M 540 are localized in myoglobin-attracting phospholipid domains of the outer membrane. Dependence of binding constant (K_m) on ionic strength is indicative of an important role of coulombic interactions in this process. Since the affinity of MbO_2 and metMb to mitochondria does not depend on overall protein charge, which varies within the pH range of 6-8, these electrostatic interactions are local, encompassing charged groups of a distinct area of the myoglobin molecule. Analysis of myoglobin 3D structure suggests that the cationic groups in the heme cavity surrounding, most likely invariant lysine and arginine residues, whose ionization state remains unchanged within the studied pH range, are implicated in electrostatic interactions with anionic groups of phospholipids.

MATERIALS AND METHODS

Materials. Mitochondria from rat liver were isolated by a standard method using differential centrifugation at 800-3000*g* in 5 mM Hepes buffer, pH 7.4, containing 220 mM mannitol, 70 mM sucrose, and 1 mM EGTA. The last centrifugation was carried out at 3000*g* (15 min) providing sedimentation of large non-fragmented mitochondria only. The freshly isolated native mitochondria with respiratory control coefficient of 4.5 through 6.5, quickly frozen at −18°C and once thawed, were used.

MetMb from skeletal muscle of sperm whale (fraction IV, 17.8 kDa) was isolated and purified as described earlier [1, 2, 4], and MbO_2 was prepared aerobically by reduction of metMb with sodium dithionite followed by separation by gel filtration on a column with Sephadex G-25.

Fluorescence probes 1,8-ANS (Serva, Germany) and M 540 (Merck, Germany), as well as EGTA (Sigma, USA), Tris (Serva), Hepes (Gerbu, Germany), d-mannitol (analytical purity grade), sucrose (extra purity grade), and KCl (extra purity grade) were used without additional purification. Phosphate buffers, KH_2PO_4, and K_2HPO_4, were recrystallized from water/ethanol solution.

The probes 1,8-ANS and M 540 were added to mitochondrial suspension containing 0.2 mg/ml of mitochondrial protein in a medium without succinate at pH 7.4 or 6.4 up to the final concentration of 0.8 and 0.16 μM, respectively.

Methods. Absorption spectra in the visible and UV ranges were recorded using a Specord UV VIS spectrophotometer (Germany). Concentration of metMb was determined using extinction coefficients ($mM^{-1}\cdot cm^{-1}$) ε_{409} = 158 and ε_{505} = 9.5, and concentration of MbO_2 using ε_{581} = 14.2 [1, 2, 4].

Flavin fluorescence of mitochondrial suspension was excited at 450 nm and recorded at 480-620 nm (emission maximum 510 nm; monochromator slit widths (excitation and emission) 7 × 6 nm). To diminish the light scattering by mitochondria, the measurements were carried out in a quartz cell, 2 × 2 mm. The sample volume was 200 μl of the medium without succinate (10 mM Tris-HCl buffer, pH 7.4 or 6.4, containing 250 mM sucrose, 0.5 mM EGTA, and 5 mM KH_2PO_4). Concentration of mitochondrial protein was 0.2 mg/ml. The quenching of flavin fluorescence from the mitochondria by MbO_2 and metMb was studied under the same conditions.

Tryptophan fluorescence of mitochondrial suspension was recorded on a Perkin-Elmer MPF-44B spectrofluorimeter (USA) in the spectral range of 300-420 nm (excitation 286 nm, emission maximum 330 nm, excitation and emission slit widths 4 × 4 nm). The quenching of tryptophan fluorescence of mitochondria by MbO_2 and metMb was studied under the same conditions.

Fluorescence spectra of the probe 1,8-ANS were recorded at 420-540 nm (excitation 360 nm, emission maximum 470 nm, excitation and emission slit widths 5 × 5 nm). The quenching of 1,8-ANS fluorescence by MbO_2 and metMb was studied under the same conditions.

Fluorescence of M 540 was excited at 540 nm and recorded at 560-620 nm (emission maximum 585 nm, excitation and emission slit widths 6 × 5 nm). The quenching of M 540 fluorescence by MbO_2 and metMb was studied under the same conditions.

In all cases, the Raman band of the buffer was subtracted from the fluorescence spectra. Emission spectra were corrected for shielding and reabsorption caused by myoglobin, as well as for dilution of mitochondrial suspension, according to the following equation:

$$F_{corr} = F_{obs}/(T_1T_2V),$$

where F_{corr} is the corrected fluorescence in the spectral maximum, F_{obs} is observed fluorescence intensity, T_1 is transmission of myoglobin at the excitation wavelength, T_2 is transmission of myoglobin at the emission maximum, and dilution $V = V_{in}/V_{fin}$.

RESULTS

Intrinsic flavin and tryptophan fluorescence of mitochondrial suspension is not quenched by metmyoglobin at pH 6-8 (Fig. 1, a and b). Similarly, MbO_2 also does not quench flavin and tryptophan fluorescence of mitochondria in this pH range (data not shown).

In contrast, MbO_2 efficiently quenches emission of the 1,8-ANS probe associated with mitochondria (Fig. 2a). Figure 2b demonstrates the plot of corrected intensity of 1,8-ANS fluorescence in the spectral maximum (F_{corr}) vs. MbO_2 concentration at pH 7.4 (quenching degree 24.5%), and inset in Fig. 2b – constant of MbO_2 binding with mitochondria, K_m, calculated from this plot and equal $2.2 \cdot 10^4$ M^{-1}. Similar data with MbO_2 as a quencher were obtained at pH 6.4 (Table 1). In both cases, similar values of 1,8-ANS fluorescence quenching degree and K_m of oxymyoglobin are observed, which apparently do not depend on pH in the range of 6-8.

Metmyoglobin also efficiently quenches fluorescence of mitochondrion-associated 1,8-ANS (Table 1). When metMb is used as a quencher, similar K_m and quenching degree values are observed at pH 6.4 and 7.4 with no dependence on pH in the pH range of 6-8, in like

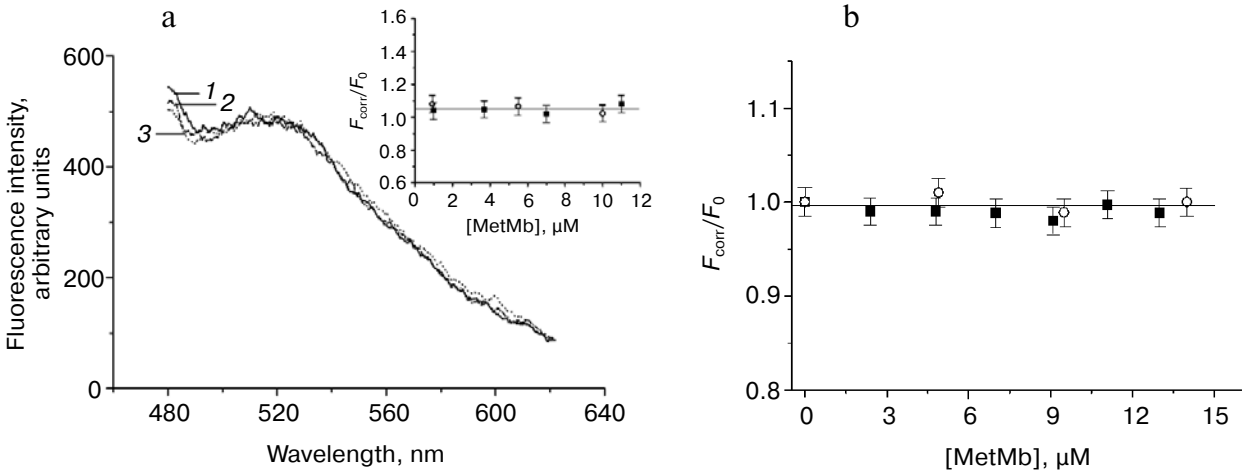

Fig. 1. a) Quenching of the intrinsic flavin fluorescence of mitochondrial suspension by metmyoglobin. Reaction medium, pH 7.4, does not contain succinate (see "Materials and Methods"). *1-3*) metMb concentration are 0, 4.8, and 9.1 μM, respectively. Inset: quenching the flavin fluorescence of mitochondria by metMb. Reaction medium, pH 6.4 (light dots) and pH 7.4 (dark dots), does not contain succinate. Excitation wavelength 450 nm, spectral maximum 510 nm, monochromator slit widths (excitation and emission) 7 × 6 nm. b) Quenching of the intrinsic tryptophan fluorescence of mitochondria by metmyoglobin. Reaction medium, pH 6.4 (light dots) and pH 7.4 (dark dots), does not contain succinate. Excitation wavelength 286 nm, emission maximum 330 nm, monochromator slit widths (excitation and emission) 4 × 4 nm.

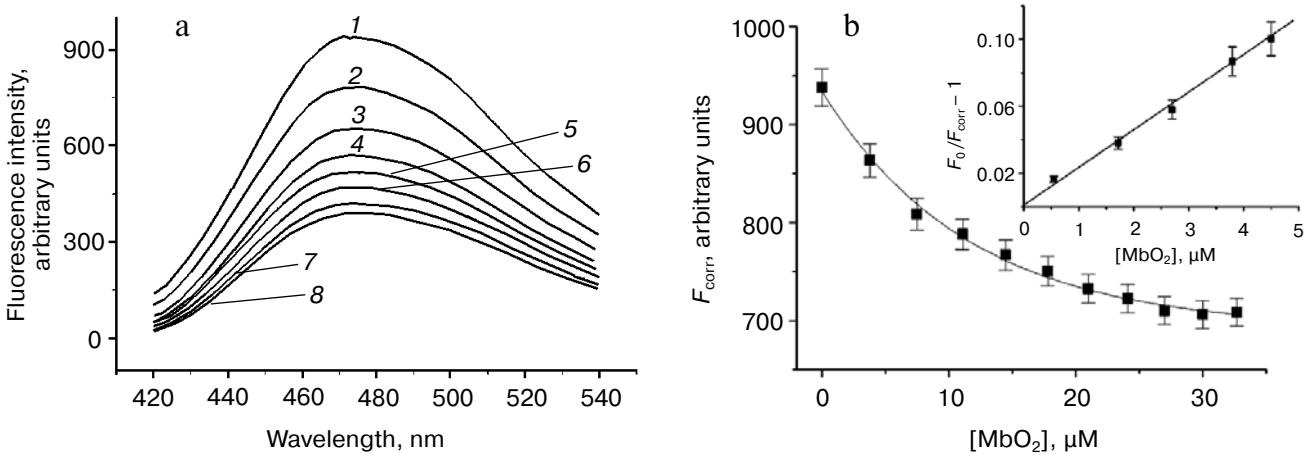

Fig. 2. a) Quenching of the fluorescence of mitochondrion-associated 1,8-ANS probe by oxymyoglobin. *1-8*) MbO_2 concentrations are 0, 3.8, 7.5, 11.1, 14.5, 17.8, 21.0, and 24.1 μM, respectively (medium without succinate, pH 7.4). Excitation wavelength 360 nm, emission spectrum maximum 470 nm, monochromator slit widths (excitation and emission) 5 × 5 nm. b) Plot of corrected fluorescence intensity of 1,8-ANS in spectral maximum (F_{corr}) vs. MbO_2 concentration at pH 7.4. Inset: determination of the constant of MbO_2 binding with mitochondria in the quenching complex.

Table 1. Quenching of the mitochondrion-associated 1,8-ANS and M 540 probes by oxy- and metmyoglobin at pH 6-8 (medium without succinate)

Ligand form of Mb	pH 7.4		pH 6.4	
	$K_m \times 10^{-4}$, M^{-1}	Quenching degree, %	$K_m \times 10^{-4}$, 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 2. Constants of metMb binding with mitochondria at pH 6-8 and medium ionic strength ranging from 10 to 150 mM KCl

Ionic strength, mM	$K_m \times 10^{-4}$ (from M 540 fluorescence quenching), M^{-1}		$K_m \times 10^{-4}$ (from 1,8-ANS fluorescence quenching), M^{-1}	
	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

manner as for 1,8-ANS fluorescence quenching by MbO_2. Although the 1,8-ANS quenching degrees are nearly equal (within the experimental error) for both myoglobins, the constant of metMb binding with mitochondria is about 1.5 times greater than the corresponding constant for MbO_2.

Fluorescence of the lipid probe M 540 associated with mitochondria is also efficiently quenched by both MbO_2 and metMb at pH 6-8. Figure 3 demonstrates the data on M 540 fluorescence quenching by oxymyoglobin at pH 7.4. Similar data with MbO_2 as the quencher is also obtained at pH 6.4 (Table 1). In both cases, similar M 540 fluorescence quenching degrees and quencher binding constant values are observed, which, hence, do not depend on pH.

Table 1 also presents the data on M 540 fluorescence quenching by metmyoglobin at pH 6.4 and 7.4. Degrees of M 540 emission quenching by MbO_2 and metMb are nearly equal, suggesting equal accessibility of the probe for both myoglobins. However, the constant for metMb binding with mitochondria determined from the data on M 540 emission quenching is about 1.5 times higher than that for MbO_2 binding, like in the case of the 1,8-ANS probe.

When 1,8-ANS and M 540 fluorescence is quenched by metmyoglobin in the presence of KCl varying in concentration in the range from 10 to 150 mM, one can observe a decrease in the constant of metMb binding with mitochondria (Fig. 4) suggesting an important role of electrostatic interactions in the quenching complex formation. Note that K_m values at pH 7.4 and 6.4 are equal within the experimental error for each ionic strength (Table 2), i.e. they do not depend on the overall protein charge, which varies within the pH range of 6-8.

DISCUSSION

The heme group of myoglobins is submerged into the protein globule rather than exposed on its surface, so the quenching of donor emission by myoglobin cannot occur by a dynamic mechanism. Energy transfer to the heme only occurs in a complex whose lifetime exceeds that of donor fluorescence (static quenching). The critical distance of fluorescence resonance energy transfer FRET (Forster radius) in tryptophan–myoglobin heme and flavin–heme pairs is rather large (~3.7 and ~5.0 nm, respectively) [3].

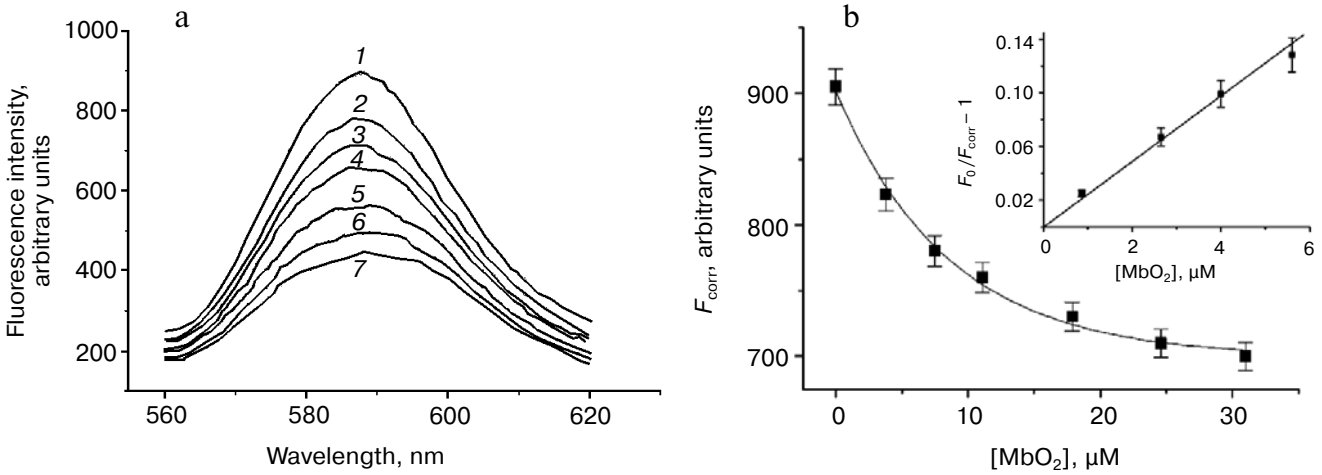

Fig. 3. a) Quenching of the mitochondrion-associated M 540 probe by oxymyoglobin. *1-7*) MbO_2 concentrations are 0, 3.8, 7.5, 11.1, 17.9, 24.6, and 31 μM, respectively (medium without succinate, pH 7.4). Excitation wavelength 540 nm, spectral maximum 585 nm, monochromator slit widths (excitation and emission) 6 × 5 nm. b) Plot of corrected M 540 fluorescence intensity in spectral maximum vs. MbO_2 concentration (pH 7.4). Inset: determination of the constant of MbO_2 binding with mitochondria in the quenching complex.

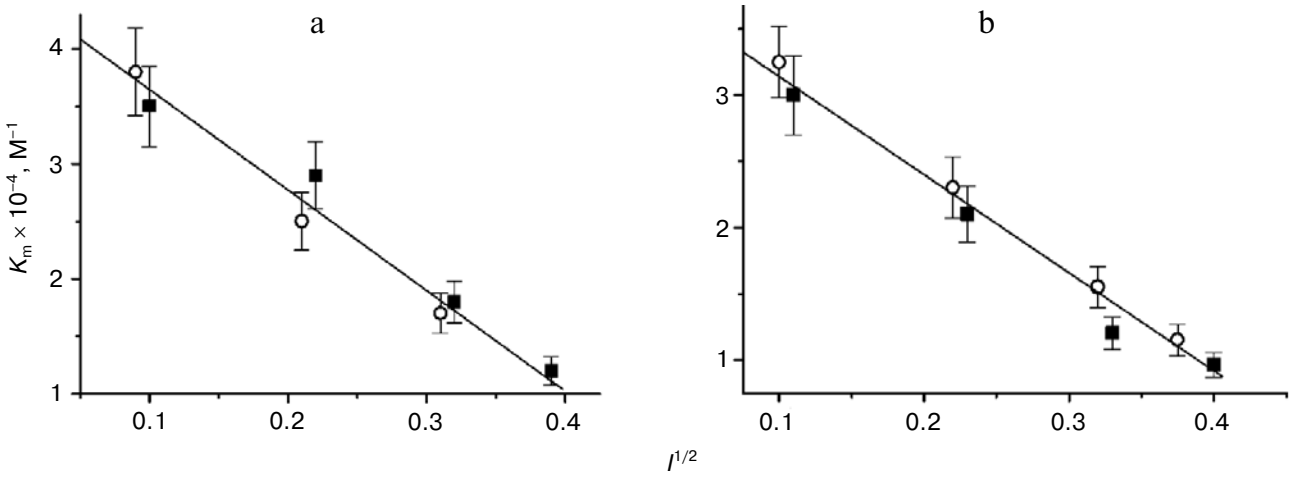

Fig. 4. Plot of the constant of metMb binding with mitochondria vs. ionic strength in the quenching complexes with 1,8-ANS (a) and M 540 (b) at pH 6.4 (light dots) and 7.4 (dark dots). Experimental conditions are the same as in Figs. 2 and 3.

Since flavins are cofactors of electron transport chain proteins of the inner mitochondrial membrane, the absence of flavin fluorescence quenching suggests that neither MbO_2 nor metMb contact the inner membrane, apparently because the outer mitochondrial membrane is impenetrable for both myoglobin and other proteins. These data suggest intactness of the studied mitochondria.

The absence of tryptophan fluorescence quenching of mitochondria suggests that mitochondrial proteins occupying ~50% outer the membrane surface do not form sufficiently tight complexes with myoglobin. It was reported earlier [3] that the energy transfer from tryptophans of proteins to the fluorescence probe is absent when the probe is localized in the membrane and the protein does not bind with the membrane. In myoglobin itself the emission of both tryptophan Trp7(A5) and Trp14(A12) located at the distances of 2.15 and 1.5 nm, respectively, from the heme is virtually completely quenched by the heme complex. The quantum yield of tryptophan fluorescence (q) in the holoprotein is only 1-5% of that in water [5].

The quantum yield of 1,8-ANS fluorescence drastically (by tens of times) increases upon binding with lipids or hydrophobic domains of proteins, and the spectral maximum shifts to shorter wavelength (in water q of the probe is only 0.004 with emission maximum at 520 nm). Most researchers suppose that in mitochondria and submitochondrial particles 1,8-ANS probe most likely binds

to specific phospholipid clusters [3]. This view is supported by the fact that polarization of the probe fluorescence in mitochondria and submitochondrial particles is rather low ($P = 0.19$) and increases about twofold ($P = 0.3$-0.4) when the probe is embedding into hydrophobic cavities of proteins [3]. When 1,8-ANS is bound to mitochondria or lipid mono- and bilayers, $q = 0.1$-0.3 and lifetime is 6-9 nsec [3], whereas these values are 0.9 and 16.5-18.7 nsec when 1,8-ANS is embedded into the hydrophobic heme cavity of various apomyoglobins [5].

At high (up to 1 mM) 1,8-ANS concentrations in solution two binding site types are found on mitochondrial membranes: high-affinity ($K_d = 2.5 \cdot 10^{-7}$ M) and low-affinity ($K_d = 1.8 \cdot 10^{-4}$ M); however, at low 1,8-ANS concentrations used in this work only the high-affinity sites are saturated, which amount is 4 nM per mg mitochondrial protein [3, 6]. Under these conditions the fluorescence spectrum only corresponds to the bound probe, and the membrane structure remains virtually unchanged.

M 540 is a member of a large family of polyene dyes widely used as fluorescence probes of membranes [3]. Upon binding with phospholipids, three M 540 forms are interconvertible: fluorescent aqueous monomer (emission at 565 nm), fluorescent membrane-associated monomer (emission at 580 nm), and membrane-associated non-fluorescent dimer [7]. At low concentration used in this work ([M 540] < 0.1 μM) all of the dye is bound and dimerization is not observed.

The presumed location of fluorescence probes 1,8-ANS and M 540 in a phospholipid bilayer is shown in Fig. 5 [3, 6, 8]. Since the charged sulfonyl group ($pK_a \sim 1$) remains on the surface, the 1,8-ANS molecule cannot deeply submerge into the bilayer and is disposed so that one side of the phenyl ring juxtaposes the glycerol moieties of phospholipids, while the other contacts water (Fig. 5a). The M 540 molecule is composed of an unsaturated hydrocarbon chain and two ring systems and also contains the negatively charged sulfonyl group (Fig. 5b). So, M 540 is located in the intermediate area of polar heads of phospholipid bilayer with the sulfonyl group oriented towards the more polar outer surface of the heads and the other part of the rod-shaped M 540 molecule between phospholipid ester bonds, being anchored in the hydrocarbon tail area with two butyl groups [8]. Like 1,8-ANS with charged sulfonyl group, M 540 cannot easily penetrate across the mitochondrial membrane (in any case, does not penetrate during the time of the experiments) [6, 9].

The fact that fluorescence of 1,8-ANS and the lipid probe M 540 is equally well quenched by MbO_2 and metMb and quenching parameters (quenching degree and quencher binding constant) are very similar in the two cases (Tables 1 and 2) suggests that both probes are localized in phospholipid domains of the mitochondrial membrane and these domains are the sites myoglobin binds to. The lifetime of quenching complex with 1,8-ANS exceeds the lifetime of the probe fluorescence, 6-9 nsec (emission lifetime of M 540 is about 2 nsec [7]). Mild fluorescence quenching (by 30 ± 10%) of bound probes, which, as mentioned above, do not penetrate into mitochondria, is likely because not all bound probe molecules form a complex with quencher due to either heterogeneity of binding sites or improper mutual orientation of donor and acceptor.

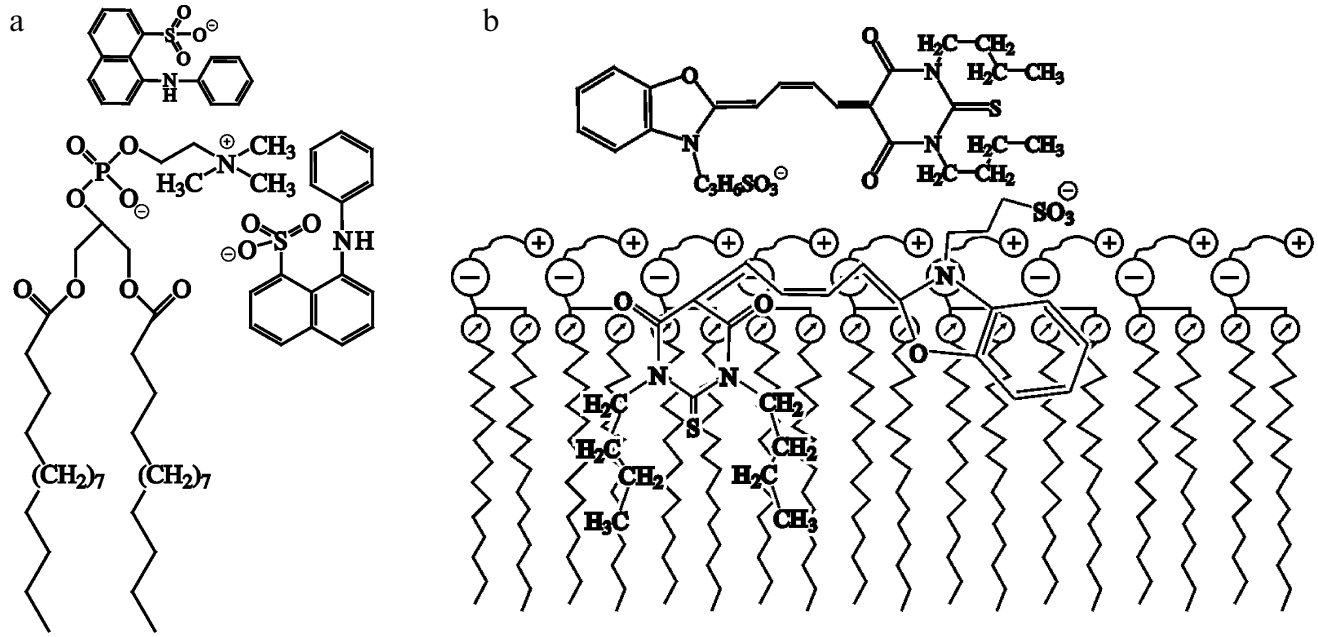

Fig. 5. Presumed disposition of 1,8-ANS (a) and M 540 lipid probe (b) molecules in phospholipid membrane [3, 6, 8].

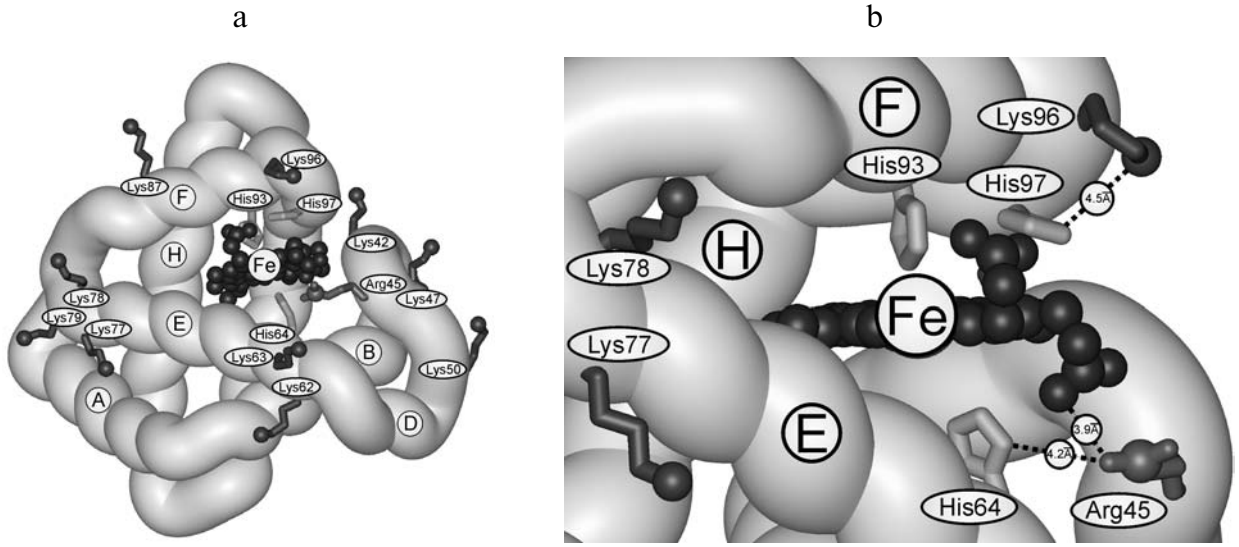

Fig. 6. a) Spatial structure of ligand-free sperm whale myoglobin and localization of invariant lysine and arginine residues in the environment of the heme cavity. The image was rendered using MOLMOL v2k2 and Corel DRAW v.11 software using the sperm whale myoglobin atomic coordinates from Protein Data Bank (PDB:1VXA). b) Fragment of myoglobin structure in vicinity of heme. The proximal His93(F8) bound with the Fe atom of the heme and distal His64(E7) forming a hydrogen bond with the O_2 ligand in the heme cavity are shown.

Coulombic electrostatic interactions seem to significantly contribute to the myoglobin–mitochondrion complex formation because a clear dependence of K_m on ionic strength is observed (Fig. 4). Since the affinity of both MbO_2 and metMb to the mitochondrial membrane remains virtually unchanged in the pH range of 6-8, in which the overall protein charge changes significantly, the overall protein charge does not influence the binding of myoglobin to the mitochondria. So, the effect of ionic strength on affinity of myoglobin to the mitochondrial membrane should be determined by local electrostatic interactions of polar groups localized in a distinct area of the myoglobin molecule.

The fact that affinity of metMb to the membrane is about 1.5-fold higher than the affinity of MbO_2 (Table 1) suggests that the charged groups surrounding the heme cavity of myoglobin can be implicated in the interaction with the mitochondrial membrane. The difference in MbO_2 and metMb binding to the mitochondrial membrane cannot be explained by ligand-induced conformational changes of the protein structure, because they are very small and primarily confined to the site of ligand attachment at the distal part of the heme cavity. One can suppose that a certain role here belongs to the difference in electron and charge states of the heme complexes, namely the difference between diamagnetic neutral ferroheme in MbO_2 (the Fe atom ligand is O_2) and paramagnetic charged ferriheme in metMb (charge +1; the Fe atom ligand is H_2O).

Many years ago researchers noticed the fact that a sequence composed of seven invariant charged amino acid residues exists on the surface of all myoglobins in the vicinity of heme near the bend between helices C and D (Fig. 6a). These amino acids are supposed to serve for binding of myoglobin to mitochondria or other cell structures [10, 11]. Both our data and analyses of myoglobin structure suggest that coulombic interactions between phospholipid polar groups (heads), whose zwitterionic character is clear at pH 6-8, and oppositely charged myoglobin groups near the heme cavity, whose ionization state remains unchanged within this pH range, contribute to complex formation between myoglobin and a mitochondrion. The most likely candidates for interacting with the anionic phospholipid groups are invariant lysine and arginine residues localized in environment of the myoglobin heme cavity (Fig. 6).

The data obtained using fluorescence probes 1,8-ANS and M 540 are in good agreement with data obtained earlier by others [1, 2, 12]. The constant of MbO_2 binding with mitochondria ($\sim 10^4$ M^{-1} at $I = 0.15$) and lifetime of the complex (tens of nanoseconds) correspond to low affinity of myoglobin to mitochondrial membrane, which is optimal for its efficient functioning as an oxygen transporter.

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