Study of Interaction of Fluorescent Cytochrome *C* with Liposomes, Mitochondria, and Mitoplasts by Fluorescence Correlation Spectroscopy

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Abstract—Here, we studied the interaction of Cys-substituted (G56C) cytochrome *c* labeled with sulfocyanin-3 fluorescent dye (fCyt) with artificial and natural lipid membranes by using fluorescence correlation spectroscopy (FCS). Compared to mitochondria, mitoplasts were shown to have more fCyt binding sites with a lower affinity for this protein. The fCyt affinity for cardiolipin-containing liposomes depended on the content of cardiolipin in liposomes and decreased upon raising the ionic strength of the solution. A high value of the constant of the fCyt binding with mitochondria could be explained by the presence of specific binding sites for this protein on the mitochondrial outer membrane surface. This explanation is confirmed by observation of more efficient displacement of fluorescent cytochrome *c* by the unlabeled mutant variant K8T than by the WT protein, whereas in mitoplasts and liposomes the WT protein displaced fCyt more efficiently than K8T.

INTRODUCTION

The heme-containing protein cytochrome *c*, one of the most important components of the electron transport, or respiratory, chain (ETC) of mitochondria, transfers electrons from ubiquinol-cytochrome *c* oxidoreductase (complex III) to cytochrome *c* oxidase (complex IV). Along with participation in electron transport, another one of its important functions is the initiation of cell apoptosis through participation in the formation of the apoptosome [1]. At the moment, significant efforts are directed toward the study of cytochrome *c* as a trigger and mediator of apoptotic events.

Programmed cell death, or apoptosis, is extremely important for the normal functioning of the body as a whole. During the activation of the cascade of events leading to cell apoptosis, cytochrome *c* is translocated through the outer mitochondrial membrane into the cytosol, where it either enhances the external apoptotic signal or initiates the activation of the caspase cascade via its own (cytochrome *c*-dependent) apoptotic pathway. The key event in the cytochrome *c*-dependent apoptotic pathway is the permeabilization of the outer mitochondrial membrane, which results in various apoptogenic factors, including cytochrome *c*, entering the cytosol. In this regard, the study of the interactions of cytochrome *c* with lipid membranes is of great interest. The interaction of cytochrome *c* with cardiolipin, a lipid that is part of the inner membrane of mitochondria, significantly enhances its peroxidase activity [2, 3]. It is assumed that by triggering the peroxidation of membrane lipids, it is the peroxidase activity that promotes translocation of cytochrome *c* across the outer mitochondrial membrane [4]. It should also be noted that cardiolipin, by itself [5] or in combination with cytochrome *c* oxidase [6], forms a membrane binding site for cytochrome *c* on the outer surface of the inner mitochondrial membrane under conditions of homeostasis as well.

It is known that the binding of cytochrome *c* with cardiolipin at the first stages is reversible, depends on pH [5], and leads to a change in the tertiary and secondary structure of the cytochrome *c* molecule [7]. A

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; CL, cardiolipin; FCS, fluorescence correlation spectroscopy; PC, egg phosphatidylcholine; fCyt, fluorescently labeled cytochrome *c*; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ETC, electron transporting chain; WT, wild type cytochrome *c*; 4Mut, mutant cytochrome *c* with the K72E/E69K/K86E/K87E substitutions.

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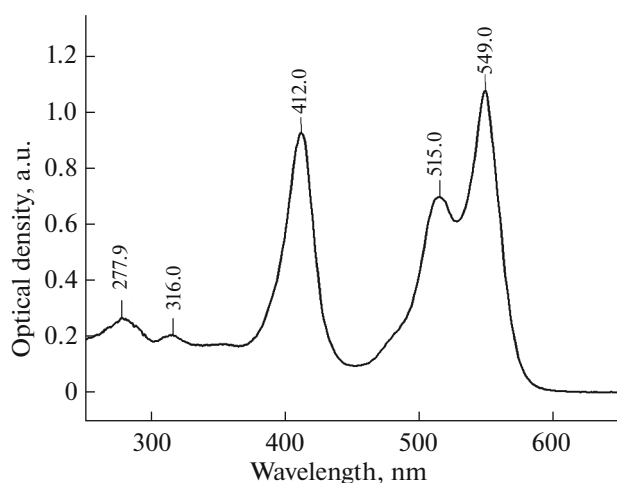
Fig. 1. Spectrum of fluorescently labeled cytochrome *c* with the G56C substitution. Absorption at 412 nm corresponds to the maximum absorption of cytochrome *c*, at 549 nm, to the maximum absorption of sulfo-cyanine.

factor affecting the stability of the cytochrome *c*—cardiolipin complex is the oxidation of cardiolipin, since cytochrome *c* has reduced affinity for the oxidized lipid [8]. At the same time, the interactions of cytochrome *c* with cardiolipin in the mitochondrial membrane remain not fully understood and attract the close attention of researchers [9–14].

The interaction of exogenous cytochrome *c* with natural (mitochondria and mitoplasts) and artificial (cardiolipin-containing liposomes) lipid membranes, as well as the effect of ionic strength and the content of cardiolipin on this process, was investigated using a fluorescently labeled variant of equine cytochrome *c* by fluorescence correlation spectroscopy in the lipid membrane.

RESULTS AND DISCUSSION

Preparation of fluorescently labeled cytochrome *c*. As a strategy for labeling cytochrome *c*, we chose the conjugation of protein molecules and a maleimide derivative of sulfo-cyanine 3 through the Cys residue introduced into the cytochrome by site-directed mutagenesis. The reaction of thiols with maleimides is widely used for bioconjugation and labeling of biomolecules, including proteins and peptides [15]; this reaction can be carried out in an aqueous medium, which greatly facilitates the procedure.

The introduction of a thiol group into a cytochrome *c* molecule can be carried out by a single substitution during mutagenesis of a surface or terminal amino acid residue for a Cys residue. The residue replaced by the Cys residue should not be involved in interactions with cardiolipin and cytochrome *c* partner proteins, or be significant for the structural and functional integrity of the protein.

On the surface of cytochrome *c* there are three cardiolipin-binding sites: binding site A, consisting of residues K72, K73, K86, K87, and R91 [16]; binding site L, consisting of residues K22, K25, K27, as well as H33, which is involved in the binding of cytochrome *c* to cardiolipin at pH < 7.0; and binding site C, containing residues N52, H26, and P44 [17]. In addition, there are sites of cytochrome *c* binding with cytochrome *c* oxidase (including aa K8, K13, K72, K86, and K87) [18], ubiquinol-cytochrome *c* oxidoreductase (including aa K7, K8, K13, K39, K72, K79, K86, K87) [19], and cytochrome *c* peroxidase (including aa K13, K27, K72, K86, K87, Q12, and Q16) [20, 21].

To introduce a fluorescent label into the cytochrome *c* molecule, we designed and obtained a mutant variant of cytochrome *c* with the replacement of the G56 residue by the Cys residue, since the functional group of the G56 residue is located on the surface of the protein globule and is not included in any of the known protein binding sites.

The labeling reaction, as well as the purification and analysis of the reaction products, was carried out according to the procedure described in the Experimental. Fractions, in which the ratio of the peaks of cytochrome *c* and sulfo-cyanine was comparable (Fig. 1), were pooled and desalted on the same column equilibrated in a salt-free buffer, after which they were analyzed spectrophotometrically.

The interaction of fluorescently labeled cytochrome *c* with lipid membranes was studied using FCS. This method is based on the registration of fluorescence fluctuations of individual particles arising as a result of Brownian motion through a small volume of space illuminated by a laser (confocal volume). FCS is traditionally used to study the processes associated with changes in the mobility of molecules and complexes: particle aggregation, interaction of fluorescent molecules with supramolecular complexes or lipid vesicles, while the range of sizes of the studied objects is very wide—from dye molecules to particles with sizes of hundreds of nanometers [22].

During the work, the analytical signal for identification of binding was the change in the number of peaks recorded in the confocal volume. The peaks of the fluorescence signal are associated with the movement of fluorescent particles through the measured volume while stirring the suspension of particles. These can be both free molecules of the labeled protein or membrane particles with several protein molecules bound to them, which are much brighter. Figure 2 shows typical recordings of the signal from a suspension of mitoplasts and a solution of labeled cytochrome *c* (fCyt). The binding of fCyt to mitoplasts was characterized by a large number of peaks with high fluorescence intensity (curve *1*), while background peaks of low intensity were recorded in the solution of unbound fluorescently labeled cytochrome *c* (*3*). In the mitoplast suspension in the absence of the labeled
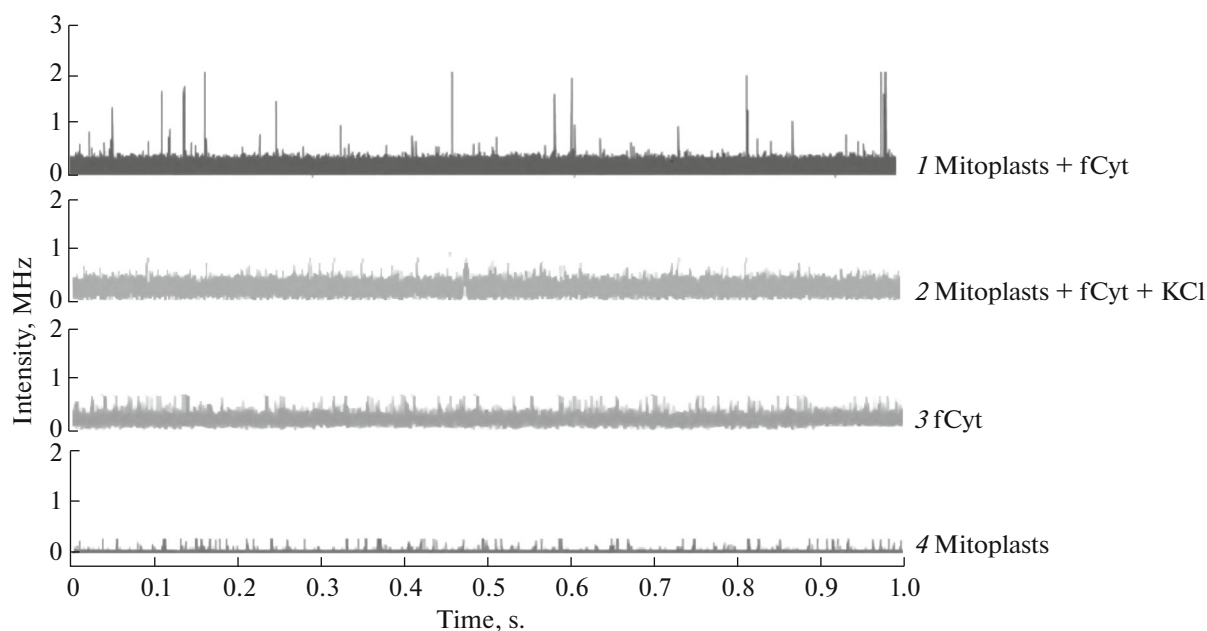
Fig. 2. Time dependences of the fluorescence intensity of a suspension of mitoplasts (*4*), a solution of labeled cytochrome *c* (*3*), suspension of mitoplasts in the presence of labeled cytochrome *c* (*1*), and a suspension of mitoplasts in the presence of labeled cytochrome *c* and 50 mM KCl (*2*). Measurements were carried out in sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4). The final concentration of mitoplasts was 0.1 mg/mL, the concentration of labeled cytochrome *c* was 0.26 μM, and the data collection time was 30 s.

protein, there were almost no peaks characteristic of the complexes (*4*), as well as in the case of the pCyt–mitoplast complex destabilized by an increase in ionic strength (*2*).

Figure 3 shows the typical distribution of fluorescence peaks and their intensity after processing the experimental data in the Saligat program. To identify binding, we chose a phenomenological intensity threshold of 1.4 MHz. Using a higher threshold value depleted the peak statistics too much, while a lower threshold value was too close to the amplifier noise, which distorted the recorded statistics.

The role of cardiolipin in the binding of cytochrome *c* to liposomes. Figure 4 shows the dependence of the binding efficiency of labeled cytochrome *c* with liposomes on cardiolipin content (from 0 to 100%). One can conclude that the efficiency of the binding of fCyt to the lipid membrane increases with an increase in the content of cardiolipin and this, apparently, is due to the fact that the number of cytochrome *c* binding sites on the surface of liposomes directly depends on the percentage of cardiolipin in the membrane and reaches its maximum value when interacting with liposomes consisting of 100% cardiolipin.

Determination of the apparent displacement constants of cytochrome *c* with different membranes. To determine the apparent displacement constants of cytochrome *c* from the complex with lipid membranes, we used the method of displacing fCyt from the complex with membranes using unlabeled cyto-

chrome *c*, counting the number of fluorescence peaks with an intensity of 1.4 MHz (Fig. 5). The displacement method is based on a decrease in the concentration of the fluorescently labeled component of the complex by replacing the unlabeled protein with an increase in the concentration of the latter in solution. The observed decrease in the number of peaks was caused by the displacement of fluorescently labeled cytochrome *c* from the complex with the studied lipid
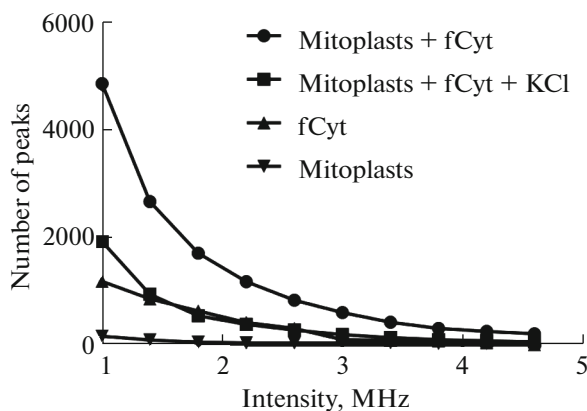
Fig. 3. Distribution of registered peaks of fluorescence by intensity in a suspension of mitoplasts and a solution of labeled cytochrome *c*, as well as upon the interaction of labeled cytochrome *c* with mitoplasts, including under conditions of increased ionic strength of 50 mM KCl. The measurement conditions are given in the caption to Fig. 2.
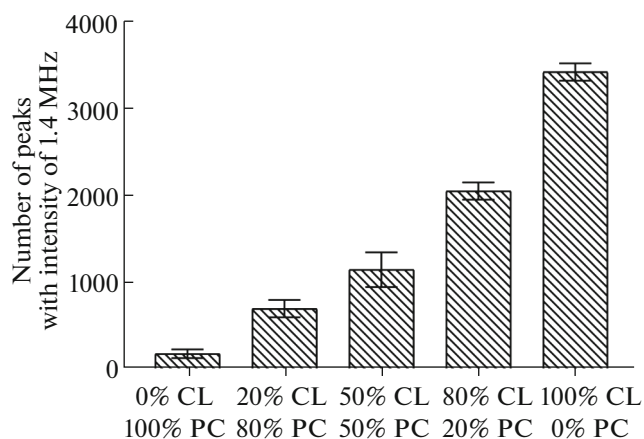
Fig. 4. Influence of the content (%) of cardiolipin in liposomes on the affinity of cytochrome *c* thereto, expressed in the number of peaks with an intensity of 1.4 MHz. Data are given for liposomes of the composition "egg phosphatidylcholine—cardiolipin" at various ratios. Measurements were carried out in sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4). The final concentration of liposomes of various compositions was 0.5 mg/mL, the concentration of labeled cytochrome *c* was 0.26 μM.

membrane as the concentration of unlabeled cytochrome *c* in the medium increased from 10 nM to 100 μM. The number of peaks was counted using the Saligat software, the displacement curves were analyzed using the GraphPrism 8 software based on the equation $y = (B_{max}X^H)/(K_b^H + X^H)$, and the main binding characteristics were determined (see Experimental): the maximum number of fluorescence peaks (B_{max}), the displacement constant (K_b); and the binding cooperativity (Hill coefficient, H). The maximum number of fluorescence peaks B_{max} reflects the concentration of fluorescent particles in the system, and also depends on the number of fCyt binding sites on one particle. In this equation, X is the cytochrome *c* concentration. The displacement constant reflects the average value of the dissociation constant of all binding sites which the labeled cytochrome *c* could bind to. Thus, the differences in the displacement constants

are due to the difference in the affinity of lipid membranes for cytochrome *c*. Hill coefficient H reflects the effect of an increase (positive cooperativity) or a decrease (negative cooperativity) in the affinity of binding sites on lipid membranes during the process of protein binding. It should be noted that this equation is applicable for analysis under two conditions: (1) the concentration of fCyt should be much higher than the concentration of binding sites; (2) saturation of binding should be ensured at the selected concentrations of fCyt and membranes. During our experiments, these conditions were met only partially, therefore, instead of the true binding constants in the experiments, the apparent binding constants were estimated. The apparent displacement constant in the case of the complex of fCyt with mitoplasts significantly exceeded those for the complexes with mitochondria and amounted to 25 μM (Table 1). In this case, the maximum number of registered fluorescence peaks in the case of the complex with mitoplasts was greater than for the complexes with mitochondria. Since the concentration of mitochondrial and mitoplast particles in our experiments was the same, these data indicate that the mitoplast membranes have a significantly greater number of fCyt binding sites. However, the differences in K_b values indicate that the affinity of the protein for binding sites on mitoplasts is about ten times lower than the affinity for similar sites on mitochondria.

The effect of ionic strength on the apparent displacement constant (K_b) and binding cooperativity (H, Table 1) was also studied. Analysis of the data obtained allows us to conclude that, in the presence of KCl, the apparent displacement constant increased, which indicates a decrease in the affinity of cytochrome *c* for lipid membranes. The number of peaks with intensities >1.4 MHz decreased with increasing ionic strength of the solution. In the case of mitochondria and liposomes, the addition of 50 mM KCl resulted in a manifold increase in the apparent displacement constant of K_b from 2 to 29 μM (mitochondria) and from 17 to 88 μM (liposomes, Table 1). The effect of KCl on K_b was significantly weaker in the case of mitoplasts. A decrease in the binding of fCyt to car-

Table 1. Values of the maximum number of peaks (B_{max}), Hill coefficient (H), and apparent equilibrium binding constant (K_b) for unlabeled wild-type cytochrome *c* (WT) obtained by processing of titration curves for each type of lipid membranes in the presence and in the absence of 50 mM KCl

Lipid membrane	Number of peaks, B_{max}	Hill coefficient, H	Displacement constant, K_b, μM
Mitoplasts	3200 ± 100	−0.5 ± 0.1	25 ± 5
Mitoplasts + KCl	2220 ± 30	−0.6 ± 0.1	67 ± 15
Liposomes (20% cardiolipin)	1700 ± 200	−0.47 ± 0.15	17 ± 2
Liposomes (20% cardiolipin) + KCl	1240 ± 50	−6 ± 0.1	88 ± 20
Mitochondria	1750 ± 150	−0.4 ± 0.1	2 ± 0.5
Mitochondria + KCl	1800 ± 50	−0.15 ± 0.05	29 ± 5
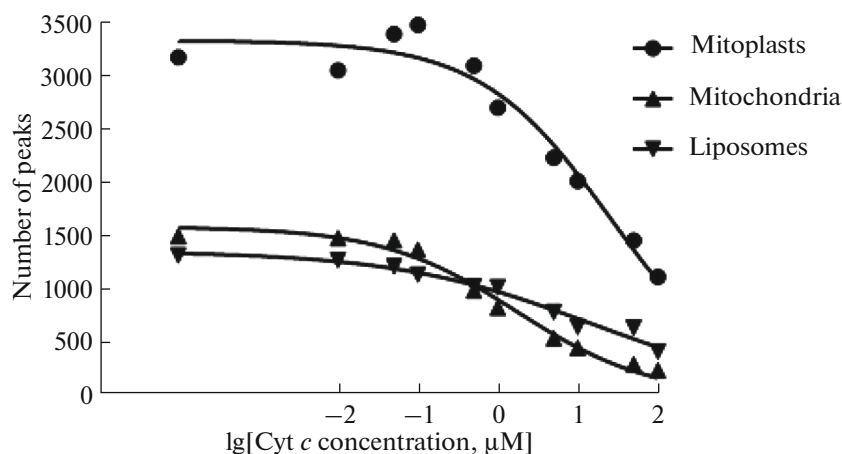
Fig. 5. Displacement of labeled cytochrome *c* from complexes with mitoplasts, mitochondria, and liposomes (20% cardiolipin) by unlabeled cytochrome *c*. The final concentration of mitoplasts, 0.1 mg/mL; mitochondria, 0.1 mg/mL; liposomes, 0.5 mg/mL; labeled cytochrome *c*, 0.26 μM. Measurements were carried out in sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4).

diolipin in liposomes with an increase in ionic strength agrees with the available literature data [22].

Determination of the apparent displacement constants of mutant cytochrome *c* with different membranes. The experiment was carried out to displace fluorescently labeled cytochrome *c* from complexes with lipid membranes using unlabeled mutant variants of cytochrome *c* with a single K8T substitution and multiple substitutions K72E/E69K/K86E/K87E (4Mut) obtained in our previous works [23, 24]. The experiment was carried out similarly to the previous experiments with titration of the fCyt–lipid membrane complex with unlabeled protein at concentrations from 10 nM to 100 μM. A characteristic drop in the number of peaks was observed (Fig. 6).

Differences in the efficiency of displacement of labeled cytochrome *c* from the fCyt–liposome complex (20% cardiolipin, 80% egg lecithin) under the effect of unlabeled mutant cytochrome *c* variants are evidenced by the different apparent displacement constants, despite the fact that the Hill coefficient is approximately the same for all cytochrome *c* variants. Based on the data presented in Table 2, it can be concluded that the mutant cytochrome *c* with the K8T substitution, having an increased affinity for cardiolipin, most effectively displaced fluorescently labeled

cytochrome *c* from complexes with cardiolipin-containing liposomes. In the case of mitoplasts, a similar picture was observed. At the same time, this protein was noticeably weaker in binding to mitochondria compared to the wild type. The mutant variant K72E/E69K/K86E/K87E (4Mut), due to the introduction of negatively charged aa residues to replace three Lys residues, exhibited lower binding than the wild-type cytochrome *c* (WT) in the case of all studied membrane types.

According to the data obtained, WT cytochrome *c* exhibited high binding efficiency to mitochondria, while the binding efficiency for 4Mut and K8T coincided and was noticeably lower compared to WT. This fact may indicate that the binding of cytochrome *c* to mitochondria did not depend on the efficiency of binding of cytochrome *c* with cardiolipin and was driven by other interactions compared to binding with mitoplasts and liposomes.

Using the FCS method, we studied the interaction of fluorescently labeled equine cytochrome *c* with three types of lipid membranes: cardiolipin-containing liposomes, mitoplasts, and mitochondria obtained from rat liver preparations. It was shown that the interaction of cytochrome *c* with mitoplasts is characterized by the highest number of binding sites, which,

Table 2. Values of Hill coefficient (*H*) and apparent displacement constant (K_b) obtained upon processing of normalized titration curves in the Prism software for unlabeled mutant cytochrome *c* variants

Cytochrome *c*	Liposomes		Mitoplasts		Mitochondria	
	Hill coefficient, *H*	K_b, μM	Hill coefficient, *H*	K_b, μM	Hill coefficient, *H*	K_b, μM
K8T	−0.45 ± 0.15	4.3 ± 1	−0.3 ± 0.15	3 ± 1	−0.4 ± 0.1	36 ± 5
K72E/E69K/K86E/K87E	−0.42 ± 0.15	1088 ± 10	−0.45 ± 0.15	52 ± 10	−0.5 ± 0.1	35 ± 5
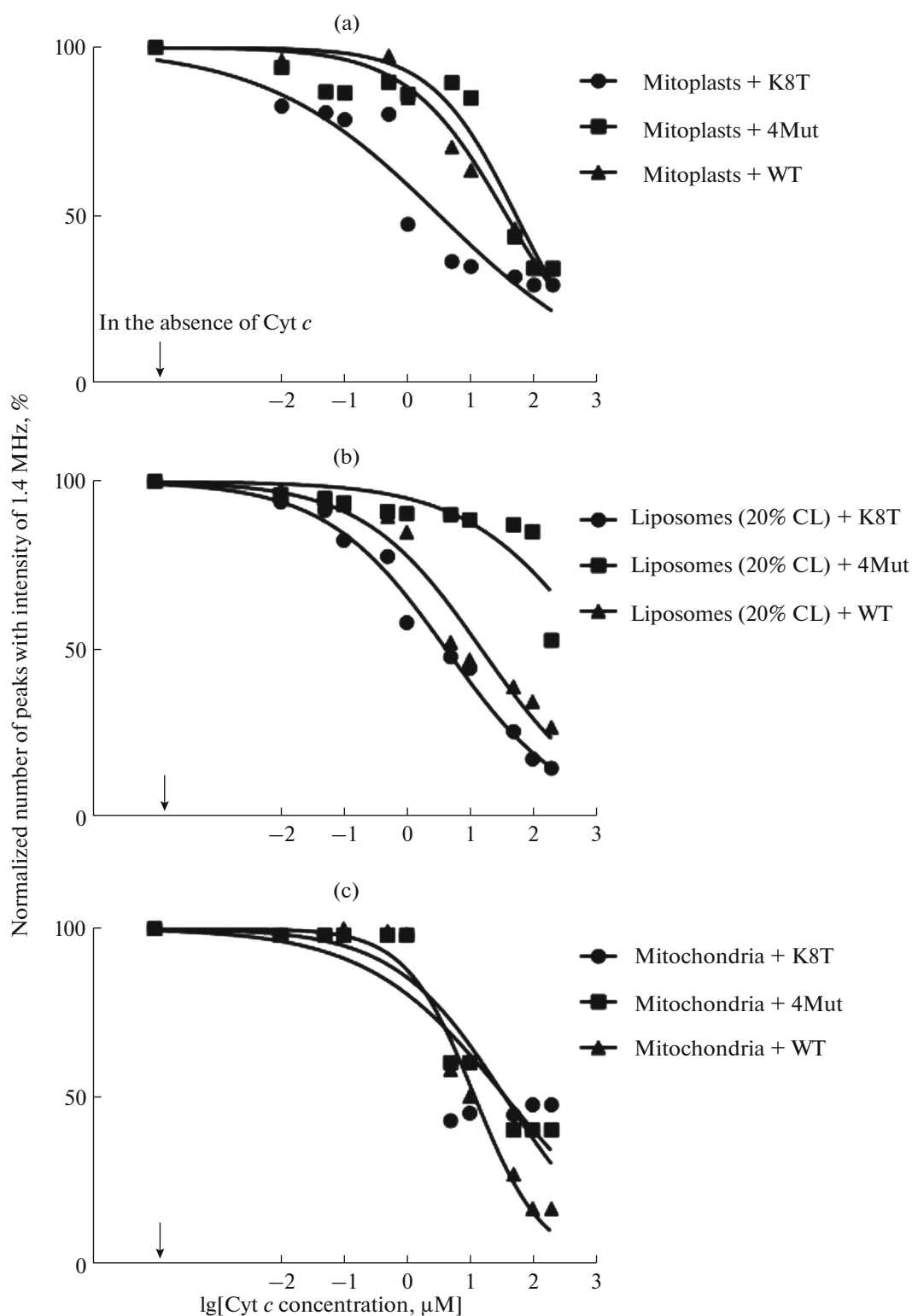
Fig. 6. Displacement of fluorescently labeled cytochrome *c* under the effect of unlabeled mutant variants K8T and 4Mut from complexes with: (a) liposomes (20% cardiolipin, 80% egg lecithin), (b) mitoplasts, and (c) mitochondria. The graphs are normalized for maximum binding. Measurements were carried out in sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4). The concentration of mitoplasts, 0.1 mg/mL; mitochondria, 0.1 mg/mL; liposomes, 0.5 mg/mL; labeled cytochrome *c*, 0.26 μM.

however, have lower affinity in comparison with mitochondria. The surface of the mitoplast membrane consists mostly of the inner mitochondrial membrane, the lipid composition of which includes cardiolipin. In addition, the inner mitochondrial membrane contains protein complexes integrated therein—ubiquinol-cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV)—which are partner proteins of cytochrome *c* within the ETC and with which cytochrome *c* forms temporary functional complexes in the process of electron transfer. Thus, mitoplasts have cytochrome *c* binding sites of at least two fundamentally different types: (1) cardiolipin, with which protein—lipid binding is implemented; and (2) complexes III and IV of the ETC, with which protein—protein binding is realized. Apparently, this explains the observed increased number of binding sites on mitoplasts in comparison with mitochondria. Both types of cytochrome *c* binding under consideration are realized with the participation of electrostatic interactions. This is consistent with our experimental observations of a decrease in the affinity of cytochrome *c* with an increase in ionic strength in the measurement medium in the case of complexes with liposomes or mitoplasts.

The monotonic increase in the affinity of fCyt to liposomes with an increase in the percentage of cardiolipin in the membrane indicates the decisive role of the interaction of fCyt with cardiolipin in the process of protein binding to the cardiolipin-containing liposomal membrane.

The nature of the binding of fCyt to the outer mitochondrial membrane (mitochondria) differed from the binding to mitoplasts and cardiolipin-containing liposomes. Nevertheless, despite the absence of cardiolipin and ETC partner proteins that bind cytochrome *c* in the outer mitochondrial membrane, stable binding was observed in our experiments. Thus, our experimental data may indicate the presence of specific cytochrome *c* binding sites in the outer mitochondrial membrane.

The results of the experiment on the displacement of fluorescently labeled cytochrome *c* from the complex with lipid membranes by mutant protein variants do not contradict the assumption that there are specific cytochrome *c* binding sites in the outer mitochondrial membrane. Both mutant variants equally efficiently (but noticeably worse than the wild-type cytochrome *c*) displaced fluorescently labeled cytochrome *c* from the complex with mitochondria. At the same time, in the case of complexes with both liposomes and mitoplasts, the variant with the K8T substitution, which has a higher affinity for cardiolipin, displaced labeled cytochrome *c* more efficiently than the K72E/E69K/K86E/K87E variant and the wild-type protein.

Our data are of interest from the point of view of understanding the fundamental interactions of cytochrome *c* with lipid membranes, as well as the influence of various external factors on these interactions. Understanding the interactions, in turn, is of practical importance since it opens up wide opportunities for the rational design of biomedical drugs with pro- or antiapoptotic properties.

EXPERIMENTAL

We used components for culture media and buffer solutions for chromatography and electrophoresis from AppliChem (Germany); ampicillin, restriction endonuclease DpnI from Fermentas (Lithuania), Pfu DNA polymerase, bovine heart cardiolipin (Sigma, United States), egg yolk phosphatidylcholine (Avanti Polar Lipids, United States), fluorescent dyes sulfocyanine and BDP TMR (maleimide form) (Lumiprobe GmbH, Germany). Distilled water was additionally purified using a Milli-Q system (Millipore, United States).

Design of mutant cytochrome *c* genes. The introduction of a mutation into the equine cytochrome *c* gene in the expression plasmid vector pBP (CYC1) was performed by site-directed mutagenesis using the QuikChangeTM Mutagenesis Kit (Stratagene, United States) [23]. After completion of the reaction, 10 units act. restriction endonuclease DpnI were added to the mixture, and the mixture was incubated for 60 min at 37°C. Then, aliquots of the resulting mixture were used to transform competent *E. coli* XL-1 Blue cells according to the standard method. The production of mutant DNA during mutagenesis was analyzed by electrophoresis in 1% agarose gel. Determination of the nucleotide sequence of mutant genes in the plasmid DNA was carried out using an ABI Prism 3100-Avant Genetic Analyzer automatic sequencer (Applied Biosystems, United States). The selected mutant genes were recloned into the expression vector pBP (CYC1) [25], modified to express the equine cytochrome *c* genes [24].

Expression of mutant cytochrome *c* genes was carried out in *E. coli* JM109 in liquid nutrient medium SB with ampicillin (final concentration 100 μg/mL) without adding an inducer at 37°C under vigorous stirring for 22–24 h [26]. Upon completion of growth, *E. coli* cells were pelleted by centrifugation at 4000 *g* at 4°C for 20 min. The resulting cell pellet was resuspended in 25 mM Na-P$_i$ buffer, pH 6.0, 1 mM NaN$_3$. Homogenization of cells was carried out under high pressure on a French Press apparatus (Spectronic Instruments, Inc., United States) followed by centrifugation at 95000 *g* for 20 min [27].

Isolation and purification of target proteins was carried out using an AKTA FPLC (GE-HEALTHCARE, United States) liquid chromatography system according to the scheme developed earlier [25, 27]. The cell extract was applied to an MP HS 10/10 cation exchange column (Bio-Rad, United States) equili-

brated with a buffer containing 25 mM Na-P_i, pH 6.0, 1 mM NaN_3. Cytochrome *c* was eluted with a linear gradient of 1 M NaCl in the same buffer at a rate of 3 mL/min. The fractions obtained after purification on MP HS were analyzed spectrophotometrically and by SDS-PAGE in 12% gel, then dialyzed against a buffer for adsorption chromatography (10 mM Na-P_i, pH 7.0, 1 mM NaN_3), and loaded onto a column with the CHT-I hydroxyapatite (Bio-Rad). Cytochrome *c* was eluted with a linear gradient of 500 mM Na-P_i, pH 7.0, at a rate of 2 mL/min. The degree of purification and the concentration of cytochrome *c* in the obtained fractions were determined spectrophotometrically and by SDS-PAGE in 12% gel. The degree of purification of cytochrome *c* in the obtained fractions was determined by the ratio of the optical density at the maximum absorption in the Soret region to the optical density at 280 nm. Fractions with the ratio of 4.5−5.0 (which corresponds to a commercial preparation purity of ≥95%, Sigma, United States) were pooled, dialyzed twice against 10 mM ammonium carbonate buffer, pH 7.9, and lyophilized on an ALPHA I-5 unit.

Preparation of fluorescently labeled cytochrome *c*. A lyophilized preparation of a mutant variant of cytochrome *c* with the G56C substitution was dissolved in a reaction buffer (10 mM NaH_2PO_4, 200 mM NaCl, pH 7.0) to a concentration of 1−2 mg/mL. The fluorescent dye, water-soluble sulfocyanine (Sulfo-Cyanine 3) in the form of maleimide, was dissolved in DMSO to a concentration (0.01 mg/mL). The dye solution was added to the protein solution at a ratio of 1 : 5, stirred, and incubated at room temperature for 12−14 h. The reaction product was purified on a gel filtration column (Sephadex G-25) equilibrated with reaction buffer. At the end of gel filtration, the fractions containing the fluorescently labeled protein were desalted on the same column equilibrated with a salt-free buffer (10 mM NaH_2PO_4, pH 7.0). The quantitative and qualitative characteristics of the modified cytochrome *c* were determined spectrophotometrically on a Cary50-Bio (Varian, United States) spectrophotometer at the absorption maxima for cytochrome *c* (412 nm) and sulfo-cyanine 3 (549 nm).

Isolation of preparations of mitochondria and mitoplasts from rat liver. Rat liver mitochondria were isolated by differential centrifugation [28] in a buffer containing 250 mM sucrose, 10 mM MOPS, 1 mM EGTA, and 0.1 mg/mL bovine serum albumin (BSA), pH 7.4. After centrifugation, the mitochondrial preparation was washed in the same buffer. Mitoplasts were obtained by incubating rat liver mitochondria for 10 min in a buffer (MOPS 10 mM, EDTA 1 mM, Tris 10 mM, pH 7.4) containing 25 mM KCl. After incubation, the suspension was centrifuged at 4200 *g* for 5 min, the supernatant was removed, and the pellet was suspended in sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4) containing 1 mM EDTA. The normalization of the con-

centrations of mitoplast and mitochondrial particles in our experiments was achieved by the fact that during the preparation of mitoplasts the same amount of mitochondrial protein was taken simultaneously into another tube, which was suspended in the same volume of buffer that was added during the last sedimentation of mitoplasts.

Preparation of cardiolipin-containing multilamellar liposomes. To prepare liposomes, 5 mg of lipid (egg lecithin, cardiolipin, or a mixture thereof) was dissolved in 250 µL of chloroform. After evaporation of chloroform under nitrogen atmosphere, 0.5 mL of buffer (Tris 10 mM, MES 10 mM, KCl 100 mM, pH 7.4) was added. The resulting mixture was shaken and then subjected to five freeze-thaw cycles. According to the given scheme, multilamellar liposomes containing 1 to 5 mg cardiolipin were obtained.

Fluorescence correlation spectroscopy. In this work, we used an instrument designed on the basis of an Olympus IMT-2 inverted fluorescence microscope (Artisan Technology Group, United States) [28]. To excite the dye, we used a 532-nm Nd: YAG solid-state laser coupled to the aforementioned microscope with a 40× magnification and a 1.2 NA water immersion objective (Carl Zeiss, Germany). The fluorescent signal passed through the appropriate dichroic separator and was projected onto the fiber core (50 µm) connected to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Canada). The signal was converted using a Flex02-01D/C interface (Correlator.com, United States). For correct operation of the device, at the beginning of the experiment, the position of the fiber was adjusted relative to the optical axis. All measurements were performed while stirring the solution with a blade-like 3-mm plastic rod rotating at 600 rpm. For measurements, BSA was added to 60 µL of sucrose buffer (180 mM sucrose, 10 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4) to a final concentration of 0.1 mg/mL and 1−3 µL of a suspension of mitochondria, mitoplasts, or liposomes. The final concentration of mitochondrial protein in the experiments was 0.2 mg/mL, and the lipid concentration in the case of liposomes was 0.5 mg/mL. The amount of fluctuations of labeled particles was measured before and after the addition of fluorescently labeled cytochrome *c* to a final concentration of 0.26 µM. The data collection time was 30 s. Fluorescence was recorded from a confocal volume located approximately 50 µm above the surface of the coverslip. Before measurements, a mixture of the labeled protein with mitochondria (as well as mitoplasts or liposomes) was incubated for 5 min.

In the experiment to displace fluorescently labeled cytochrome *c* with unlabeled cytochrome *c*, all the above steps were repeated, additionally measuring the amount of fluctuations each time after adding unlabeled protein (concentrations ranged from 10 nM to 100 µM).

To quantify the protein binding to liposomes and mitochondria, the number of fluorescence signal peaks ($n(F > F_0)$) with an intensity above a certain threshold (F_0) [22] was calculated using the WinEDR Strathclyde Electrophysiology Software developed by J. Dempster from Strathclyde University (Glasgow, UK) or a similar Saligat program developed by V. Kozlovsky (Belozersky Research Institute of Physicochemical Biology, Moscow State University). In this work, the F_0 threshold of 1.4 MHz was used to assess the binding of cytochrome c. In a typical experiment, such a threshold value allowed recording about 2000 peaks, which provided sufficient statistics and good reproducibility of measurements.

The displacement constant and Hill coefficient values were calculated by solving a parametric equation: $y = (B_{max}X^H)/(K_b^H + X^H)$, where B_{max} is the maximum number of peaks normalized for all measurements, X is the concentration of unlabeled cytochrome c, K_b is the displacement constant, and H is Hill coefficient. To fit the data with this equation, the GraphPad Prism 8 software (GraphPad Software, Inc, United States) was used. All data were obtained as a result of four independent experiments and statistically processed using the GraphPad Prism 8 (Annova test) software; the significance level of P was not less than 0.05.

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COMPLIANCE WITH ETHICAL STANDARDS

The article contains no studies involving humans as subjects of the study.

Animals were treated and the experiments were performed in accordance with all international standards on treatment and use of animals and the protocols were approved by the Ethics committee of the Belozersky Institute of Physicochemical Biology.

Conflict of Interests

The authors declare they have no conflicts of interests.

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