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Mechanism of Activation of Cytochrome *c* Peroxidase Activity by Cardiolipin

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Abstract—In this work, the actions of bovine heart cardiolipin, synthetic tetraoleyl cardiolipin, and a nonspecific anionic detergent sodium dodecyl sulfate (SDS) on cytochrome *c* (Cyt *c*) peroxidase activity recorded by chemiluminescence in the presence of luminol and on the Fe···S(Met80) bond whose presence was estimated by a weak absorption band amplitude with peak at 695-700 nm (A_{695}) were compared. A strict concurrency between Fe···S(Met80) breaking (A_{695}) and cytochrome peroxidase activity enhancement was shown to exist at cardiolipin/Cyt *c* and SDS/Cyt *c* molar ratios of 0 : 1 to 50 : 1 (by chemiluminescence). Nevertheless, when A_{695} completely disappeared, Cyt *c* peroxidase activity under the action of cardiolipin was 20 times more than that under the action of SDS, and at low ligand/protein molar ratios (≤4), SDS failed to activate peroxidase activity while cardiolipin enhanced Cyt *c* peroxidase activity 16-20-fold. A_{695} did not change on Cyt *c* binding with liposomes consisting of tetraoleyl cardiolipin and phosphatidylcholine (1 : 10 : 10), while peroxidase activity was enhanced by a factor of 8. Breaking of 70% of the Fe···S(Met80) bonds resulted in only threefold enhancement of peroxidase activity. Cardiolipin-activated Cyt *c* peroxidase activity was reduced by high ionic strength solution (1 M KCl). The aggregated data suggest that cardiolipin activating action is caused, first, by a nonspecific effect of Fe···S(Met80) breaking as the result of conformational changes in the protein globule caused by the protein surface electrostatic recharging by an anionic amphiphilic molecule, and second, by a specific acceleration of the peroxidation reaction which is most likely due to enhanced heme accessibility for H_2O_2 as a result of the hydrophobic interaction between cardiolipin and cytochrome.

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The main function of cytochrome *c* (Cyt *c*) in the cells of vertebrates is electron transfer between respiratory complexes III and IV incorporated into the inner membrane of mitochondria. A second important function of Cyt *c* has been revealed recently: coming out from mitochondria into cytosol, this mitochondrial protein initiates apoptosis (see [1-4]). A third function of Cyt *c*, according to V. P. Skulachev's hypothesis, may consist in the fact that this hemoprotein in trivalent state can act as an antioxidant oxidizing superoxide radical generated by mitochondria to molecular oxygen [2, 5]. A fourth function of Cyt *c* is that of peroxidase, i.e., catalyzing the reactions of hydrogen peroxide oxidation of organic substrates [6, 7] including lipids containing polyunsaturated fatty acids [8].

Unfortunately, the term "peroxidase activity" in regard to Cyt *c* is sometimes used when substances are oxidized by air oxygen with the formation of hydrogen peroxides. In this work, the "peroxidase" activity of Cyt *c* will imply its ability to catalyze oxidation of organic compounds in the presence of H_2O_2.

Water-dissolved Cyt *c* possesses a very low peroxidase activity that is greatly enhanced on its binding with anionic lipids on membrane surfaces. This phenomenon

Abbreviations: BCL) bovine heart cardiolipin; Cyt *c*) cytochrome *c*; DTPA) diethylenetriaminepentaacetate; PC) 1,2-dioleyl-*sn*-glycero-3-phosphocholine; SDS) sodium dodecyl sulfate; TOCL) tetraoleyl cardiolipin.

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aroused researchers' great interest since a considerable amount of Cyt *c* in mitochondria does not exist as a water-dissolved hemoprotein or in complexes with electron transport participants but as a complex with cardiolipin, which is present on the outer surface of the inner mitochondrial membrane (see reviews [9-11]). Being an activator of Cyt *c* peroxidase activity, natural cardiolipin (as well as other lipids containing polyunsaturated fatty acid chains) also turned out to be a substrate of the peroxidase reaction, which produces substances typical of lipid peroxidation such as linoleic acid monoepoxides [12] and others [11]. It was assumed that the Cyt *c*-catalyzed peroxidation of cardiolipin lies early in the succession of events resulting in cytochrome release from mitochondria and initiation of apoptosis (see reviews [10, 11]).

In spite of the fact that the phenomenon of activation of Cyt *c* peroxidase activity by cardiolipin can be considered established, the mechanism of this activation remains unclear. An evident difference in the structure of the active center of genuine heme peroxidases such as horseradish peroxidase or myeloperoxidase consist in the fact that, in Cyt *c*, all six coordination Fe^{3+} bonds are occupied (four bonds with the nitrogen atoms of the tetrapyrrole ring and two bonds with nitrogen of His18 and sulfur of Met80), while in peroxidases the bond with methionine is absent, which supposedly enables H_2O_2 to react with heme. This is why various authors draw attention to the fact that the coordinate bond between heme iron and methionine-80 sulfur (Fe···S(Met80) bond) breaks in the process of formation of cytochrome complexes with cardiolipin and other anionic lipids which can be a cause of an enhanced Cyt *c* peroxidase activity [11, 13].

The immediate breaking of the Fe···S(Met80) bond, for example, on Cyt *c* carboxymethylation, results in a significant enhancement of cytochrome peroxidase activity [14]. Nevertheless, the parallelism between a decrease in the absorption band and changes in peroxidase activity on Cyt *c* interaction with cardiolipin-containing liposomes was observed in by no means all instances [15]. In this work, the actions of bovine heart cardiolipin (BCL), synthetic tetraoleyl cardiolipin (TOCL), and a nonspecific anionic detergent sodium dodecyl sulfate (SDS) on Cyt *c* peroxidase activity recorded by chemiluminescence in the presence of luminol and on the Fe···S(Met80) bond whose presence was estimated by the weak absorption band amplitude with peak at 695-700 nm (A_{695}) were compared. The data suggest that the electrostatic interaction between anionic amphiphilic molecules and Cyt *c* breaks the Fe···S(Met80) bond and activates peroxidase activity; however, along with this, cardiolipin, but not SDS, is responsible for further multiple enhancement of peroxidase activity, which is obviously due to a specific restructuring of the macromolecule.

MATERIALS AND METHODS

Reagents. Horse heart Cyt *c* (catalog No. C7752, 99% purity) was purchased from Sigma (USA). The concentration was determined spectrophotometrically after reduction by 99% ascorbic acid (Aldrich, Germany) using the molar absorptivity at the maximum of the band (550 nm) ε_{550} = 29,400 liter/mol·cm [16]. Bovine heart cardiolipin (BCL) (98% purity), diethylenetriaminepentaacetate (DTPA), and etoposide (VP16, dimethylepipodophyllotoxin-ethylidene-glucopyranoside) were from Sigma; 1,1′,2,2′-tetraoleyl cardiolipin (sodium salt) (TOCL) and 1,2-dioleyl-*sn*-glycero-3-phosphocholine (PC) were from Avanti Polar Lipids (USA); 99% SDS was from Fluka (Switzerland). Luminol used as a chemiluminescent reagent was from ICN Biomedicals (USA) (catalog No. 195038). Hydrogen peroxide prepared by dilution of 30% H_2O_2 (Aldrich, USA) was used for investigation of peroxidase activity. Hydrogen peroxide concentration was determined spectrophotometrically using ε_{230} = 72.1 liter/mol·cm [17]. $NaH_2PO_4 \cdot 2H_2O$ (analytical grade; Khimmed, Russia) was used to prepare a phosphate buffer solution (PB), pH 7.4. KCl (analytical grade; Khimmed) solution (1 M) was used to create high ionic strength.

Preparation of liposomes. A suspension of small monolayer liposomes was obtained by ultrasonic treatment of a phospholipid suspension in 10 mM phosphate buffer + 0.9% NaCl, pH 7.4, with the addition of 100 μM chelator of heavy metals DTPA to prevent lipid autooxidation. Liposomes of two types were used: of pure PC (5 mM) and of a PC + TOCL mixture (2.5 mM each).

Cyt *c* absorption spectra were measured at room temperature on a Specord 200 spectrophotometer (Jena Eng., Germany).

The initial solutions of Cyt *c*, SDS, BCL, TOCL, luminol, and 20 mM phosphate buffer were prepared with distilled water. The total volume of the sample was 900 μl. Samples in a typical experiment contained 20 μM Cyt *c*, 200 μM luminol, SDS, BCL, or TOCL in different concentrations to create 0-, 1-, 2-, 4-, 6-, 8-, 10-, 16-, 32-, 50-, and 100-fold excesses in relation to cytochrome (mol/mol), and 20 mM phosphate buffer, pH 7.4. The mixture was shaken and left to stand for 10 min, and the absorption spectrum at 650-750 nm was then recorded relative to the luminol-containing phosphate buffer solution. It was previously shown that standing of solutions for up to 60 min did not change significantly the magnitude of the absorption. Since the peak of optical density at 695-700 nm is on the slope of the absorption band, its base line was first approximated with the straight-line equation (by the initial and final points of the peak). The obtained value was subtracted from the absorption band and the intrinsic absorption at 670-740 nm was thus determined. A_{695} was plotted against excess activator (SDS, BCL, and TOCL). The absorption band at 695-

700 nm characterizes the bond between heme iron and methionine-80 (Fe···S(Met80) bond). A decrease in A_{695} is associated with the rupture of this bond.

The Lum-01 luminometer (InterOptika-S, Russia) is a hardware and software complex intended for recording ultra-weak luminous fluxes accompanying biochemical reactions and physical and biological processes. This device allows recording the luminescence of liquid samples with a volume of 100 μl to 5 ml, control temperature of the measured samples in a range of 20 to 70°C to within 0.2°C, and stir samples at a controlled rate of 60 to 600 rpm. The intensity is measured with a highly sensitive photoelectron multiplier operating in the photon counting mode. This mode reduces noise level, enhances the device sensitivity, and extends the dynamic range of the measured signals. A threshold discriminator excludes the most part of noise signal. Light intensity expressed in pulse number is transmitted to a personal computer where it is recorded with PowerGraph software (www.powergraph.ru).

Cyt *c* peroxidase activity was investigated using the Lum-01 luminometer at room temperature. These experiments are characterized by the fact that the absorption band and peroxidase activity were investigated for one and the same sample. To perform a chemiluminescent reaction, a 150-μl sample taken from a spectrophotometric cuvette immediately after the recording of absorption spectra was put into a cuvette (see above). H_2O_2 was added to a final concentration of 30 μM, and chemiluminescence was recorded for 30 min at room temperature.

The intensity of the chemiluminescent (CL) signal was recorded in arbitrary units. Pure Cyt *c* possesses a very low peroxidase activity though on a rather great excess of cardiolipin and SDS the CL signal became almost 200-400 times greater. The Lum-01 device is characterized by a low bound of detection and a unique dynamic range of about seven orders of magnitude, which allowed recording chemiluminescence within the whole investigated range to certain accuracy. To estimate peroxidase activity, the peak in the chemiluminescence kinetic curve was used. CL signal was plotted against the ligand (SDS, BCL, or TOCL)/protein molar ratio.

It was shown that the CL response amplitude in the (Cyt *c* + SDS) system (1 : 300 mol/mol) depended linearly on the cytochrome concentration in a concentration range of 3 to 20 μM ($R^2 = 0.998$), and the amplitude grew from 0 to 450 V. The same range of cytochrome concentrations and CL signal intensities were used in further experiments. A relationship between the chemiluminescence amplitude and horseradish peroxidase concentration was investigated for comparison. The relationship was also linear within the range of 1 to 15 nM ($R^2 = 0.993$) and the amplitude grew from 0 to 600 V. Thus, horseradish peroxidase was three orders of magnitude more active than Cyt *c* in the complex with excess SDS or cardiolipin. In the absence of activating agents, Cyt *c* possessed a measurable but much lesser activity. The table and figures present the immediate chemiluminescent amplitudes that were proportional to peroxidase activity under the conditions mentioned in this work.

In one set of experiments, peroxidase activity was measured with the EPR method by the rate of etoposide radical formation. This method is presented in detail in work [15].

RESULTS

Rupture of the Fe···S(Met80) bond and enhancement of peroxidase activity under the influence of cardiolipin. Figure 1 shows the effect of nonspecific anionic detergent SDS on the absorption spectrum of Cyt *c* in the long wavelength region. It can be seen that the maximum amplitude at 695-700 nm starts to decrease at surfactant/protein molar ratio of >10 : 1 and stops decreasing at ratio of 1 : 50. When working with phospholipids, e.g. cardiolipin, or with a mixture of cardiolipin and other phospholipids, it should be born in mind that phospholipid vesicles, liposomes, are formed in the aqueous medium, and the light-scattering of samples enhances significantly. It is necessary to subtract this background in order to estimate the shape and amplitude of a rather weak absorption band of the Fe···S(Met80) bond (see "Materials and Methods"). Since the absorption in our experiments should be measured at the same, very low, cytochrome concentrations as those at which peroxidase activity was estimated, the same procedure of the treat-

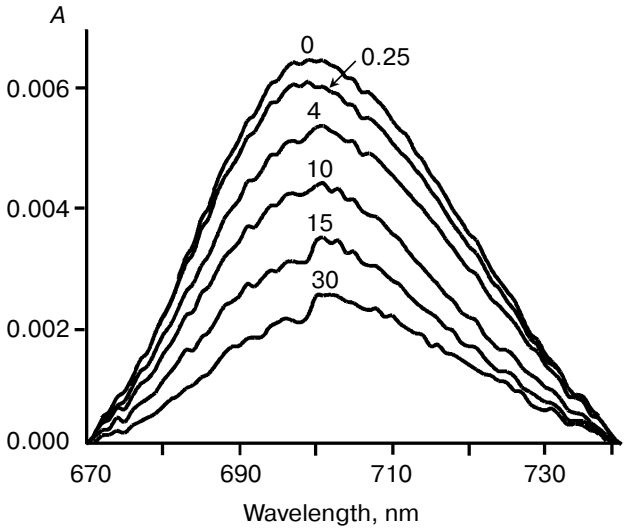

Fig. 1. Cytochrome *c* spectral absorption band in the presence of different SDS amounts after baseline subtraction; the figures by the curves show the molar ratio of SDS to Cyt *c*. Concentrations: 0.02 mM Cyt *c*, 0.2 mM luminol, up to 0.33 mM SDS, phosphate buffer solution, pH 7.4. The comparison solution contained 0.2 mM luminol in phosphate buffer solution.

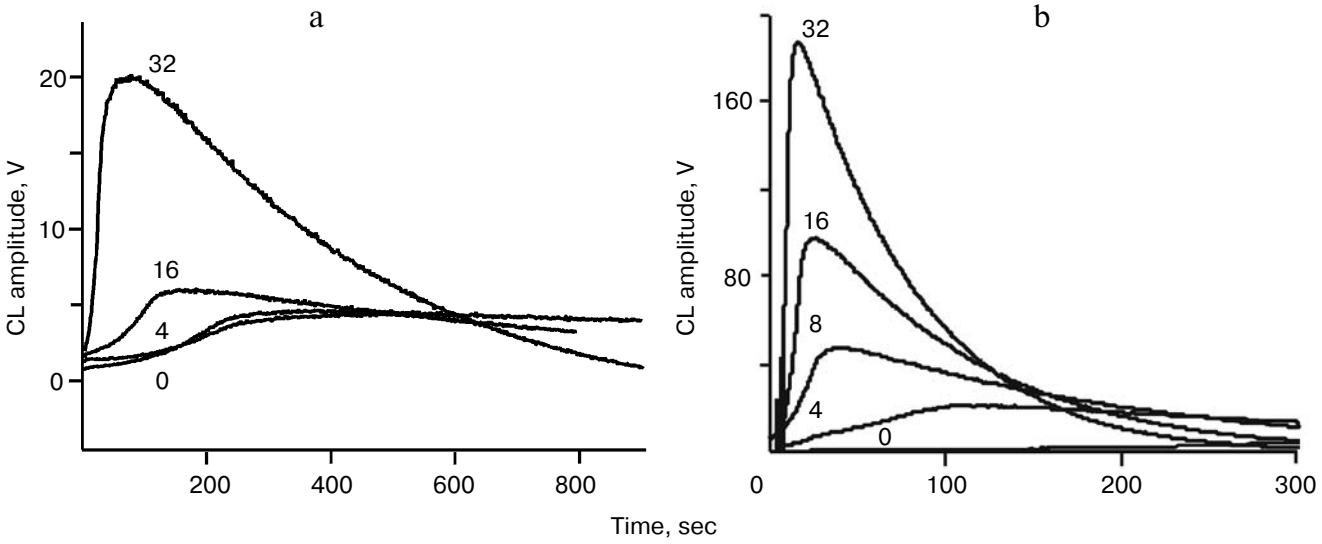

Fig. 2. Curves of chemiluminescence development at room temperature in the luminol–hydrogen peroxide system in the presence of Cyt *c* and different amounts of SDS (a) and TOCL (b); the figures by the curves show the molar ratio of SDS and TOCL to Cyt *c*. Concentrations: 0.02 mM Cyt *c*, 0.2 mM luminol, up to 0.64 mM SDS or TOCL, 0.03 mM H_2O_2, phosphate buffer solution, pH 7.4.

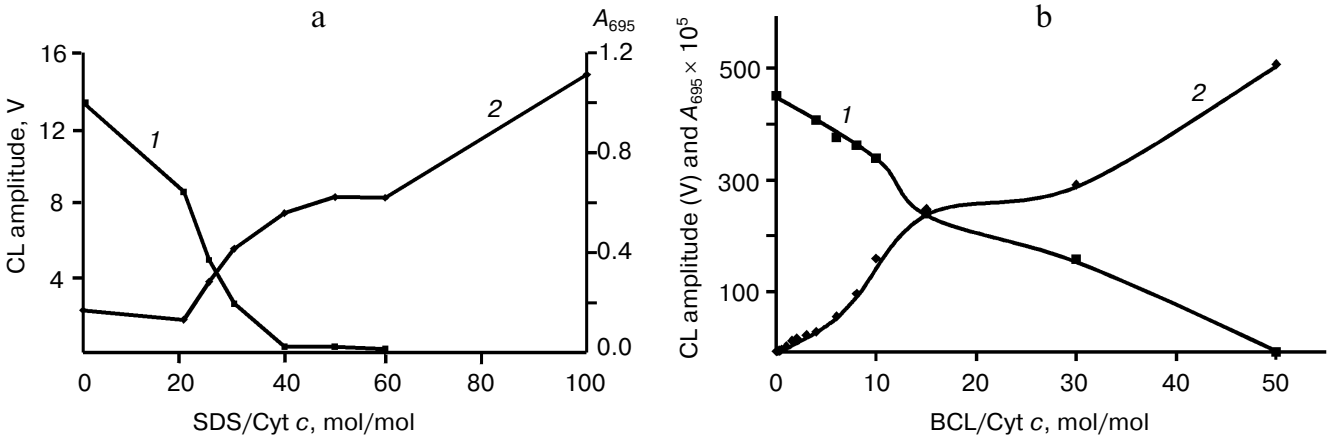

Fig. 3. a) Relative reduction in A_{695} characteristic of Fe···S(Met80) bond breaking (curve *1*) and CL signal amplitude characterizing Cyt *c* peroxidase activity (curve *2*) against the SDS/Cyt *c* ratio. b) Relative reduction of A_{695} (curve *1*) and CL signal amplitude (curve *2*) against the cardiolipin/Cyt *c* ratio. For experimental conditions, see "Materials and Methods".

ment of very weak absorption bands at 650-730 nm was carried out in the experiments with SDS. Figure 1 shows the treated in such a way absorption spectra of Cyt *c* in the presence of different amounts of SDS. It can be seen that the breaking of the Fe···S(Met80) bond in the complex was observed under the influence of the detergent. Cyt *c* peroxidase activity was activated under the influence of both cardiolipin and SDS, which can be judged by the growth of chemiluminescence amplitude in the presence of luminol (Fig. 2), simultaneously with the Fe···S(Met80) bond breaking (Figs. 3a and 3b, curves *1*).

It is interesting to compare the two effects: breaking of the Fe···S(Met80) bond and activation of cytochrome peroxidase activity. As mentioned above, the comparison was performed under strictly identical conditions: absorption spectra were first measured, and luminol and hydrogen peroxide solutions of very small volumes were then added to part of the solution to obtain practically similar concentrations of cytochrome and cardiolipin (or SDS) in the compared samples (see "Materials and Methods"). The data for SDS are shown in Fig. 3a, and those for bovine cardiolipin in Fig. 3b. It can be seen that strict parallelism between the extent of breaking of the Fe···S(Met80) bond and activation of peroxidase activity was observed in both cases. It is seen most clearly if the decrease in A_{695} band amplitude and peroxidase activity

are plotted in one scale on the ordinate: all experimental points in this case nearly fit a single curve (the data of such a scaling are not shown).

The data show that breaking of the Fe···S(Met80) bond is necessary for Cyt *c* peroxidase activity to appear. Such breaking may be equally caused by the action of both cardiolipin and the nonspecific anionic (i.e., negatively charged) detergent SDS.

Comparison of the activity of Cyt *c* in the complex with detergent and in the complex with cardiolipin. Cyt *c* peroxidase activity varies greatly under the influence of the same cardiolipin and SDS concentrations. To verify this, it will suffice to compare the figures on the ordinate in Figs. 2a and 2b. Table 1 presents the compared effects of cardiolipin and SDS on Cyt *c* peroxidase activity measured in the same experiment under similar conditions. It is seen that SDS produced no measurable activating action at low ligand/protein molar ratios (4 : 1 and 8 : 1), while cardiolipin enhanced cytochrome peroxidase activity at a ratio of 8 : 1 by a factor of 25. At ligand/protein ratio of 16 : 1 and 32 : 1, SDS activated peroxidase activity 1.5 and 7.4 times more intensively, and cardiolipin 37.7 and 94 times, respectively, and the action of both ligands on the Fe···S(Met80) bond was similar (Fig. 3). It is of interest to compare the influence of cardiolipin and SDS at the lowest concentrations when the Fe···S(Met80) bond is broken.

Table 1. Effect of bovine heart cardiolipin (BCL) and dodecyl sulfate (SDS) on cytochrome *c* (Cyt *c*) peroxidase activity

Ligand/Cyt *c* ratio	SDS	BCL	BCL + 1 M KCl
0	2.7	2.7	2.7
4	2.8		30
8	1.1	68	60
16	4.2	103	68
32	20.3	257	95

Note: Amplitudes of chemiluminescence in the presence of luminol and hydrogen peroxide are presented.

Figure 4 presents the records of chemiluminescence in the presence of H_2O_2 and luminol for Cyt *c* (curve *3*) and for its mixtures with SDS (curve *2*) and cardiolipin (curve *1*) at ligand/protein ratio of 50 : 1. Under these

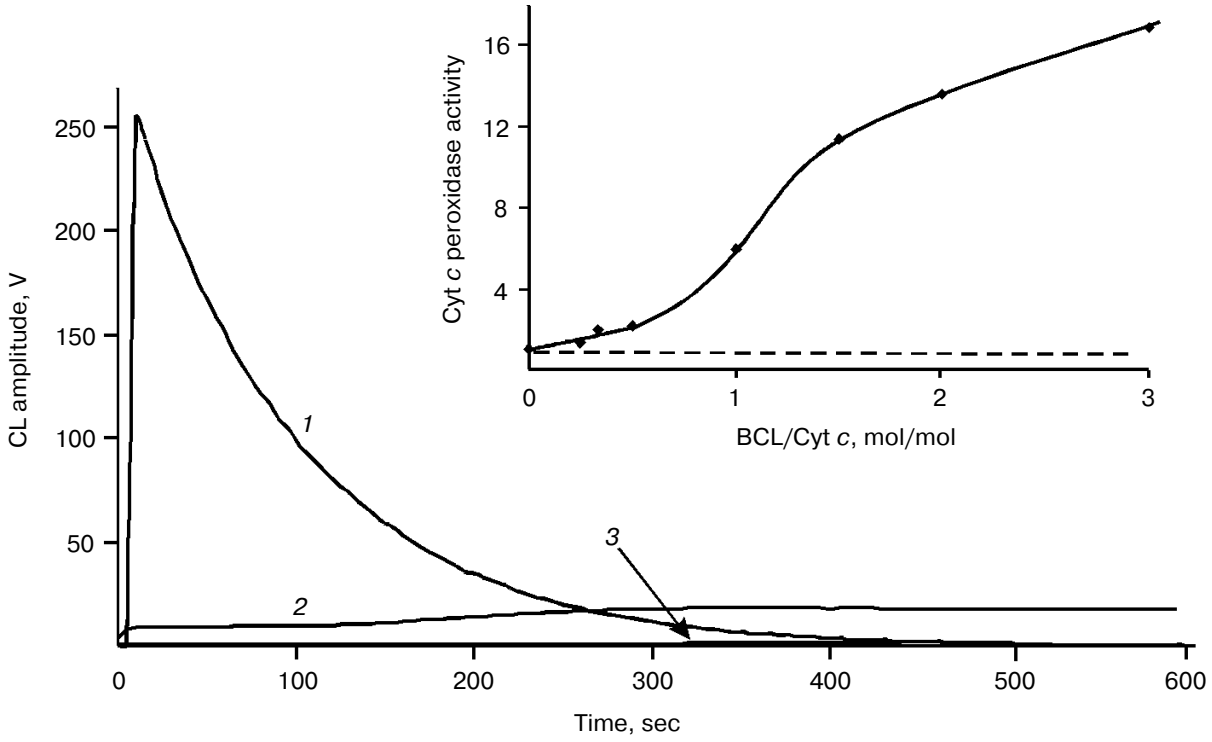

Fig. 4. Cytochrome *c* peroxidase activity in the presence of SDS and bovine heart cardiolipin (BCL). The curves of chemiluminescence development of Cyt *c* in the presence of BCL (*1*), Cyt *c* in the presence of SDS (*2*), and Cyt *c* alone (*3*). The ligand/protein molar ratio is 32 : 1. Concentrations: 0.02 mM Cyt *c*, 0.2 mM luminol, 0 to 0.64 mM SDS or BCL, 0.03 mM H_2O_2, 20 mM phosphate buffer, pH 7.4. Insert: relationship between Cyt *c* peroxidase activity and the BCL/protein molar ratio (the peroxidase activity is shown relative to the activity of Cyt *c* without cardiolipin).

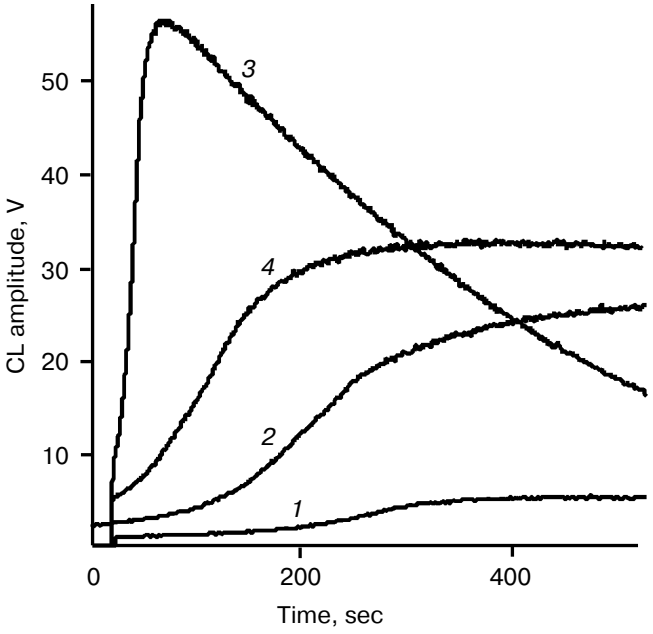

Fig. 5. Curves of Cyt *c* chemiluminescence development in the presence of different bovine heart cardiolipin (BCL) amounts in a 20 mM buffer solution and in the presence of 1 M KCl. Concentrations: 0.02 mM Cyt *c*, 0.2 mM luminol, up to 0.16 mM BCL, 0.03 mM H_2O_2, phosphate buffer solution, pH 7.4. Curves: *1*) Cyt *c*; *2*) Cyt *c* + KCl; *3*) Cyt *c* + BCL (1 : 4); *4*) Cyt *c* + BCL (1 : 4) + KCl.

conditions, when the absorption band A_{695} with both ligands disappeared, the difference in the enhancement of peroxidase activity was impressive: cardiolipin enhanced the activity by a factor of 228 while SDS by only 16. Thus, cardiolipin enhanced peroxidase activity 16 times more than SDS despite their similar action on the Fe···S(Met80) bond breaking. It is obvious that the action of cardiolipin is by no means confined to breaking the Fe···S(Met80) bond but is most probably caused by its specific effect on the molecular conformation whose probable nature will be considered below.

Role of electrostatic interactions in the activating effect of cardiolipin and nonspecific detergent SDS on Cyt *c* peroxidase activity. The experiments carried out in the laboratory headed by V. E. Kagan revealed that the activating effect of cardiolipin on Cyt *c* peroxidase activity weakened in solutions of high ionic strength [15]. In this work, values of Cyt *c* peroxidase activity were compared in the presence of cardiolipin at different concentrations in a low ionic strength buffer and in the same buffer solution containing 1 M KCl. As seen in Fig. 5 and Table 1, cytochrome peroxidase activity reduced, though relatively little, under the influence of high ionic strength. This suggests that the effect of electrostatic interactions on activation of cytochrome peroxidase activity is not great and hydrophobic interactions probably play a much greater part.

Effect of hydrogen peroxide direct breaking of the Fe···S(Met80) bond on Cyt *c* peroxidase activity. It is known that hydrogen peroxide taken in excess relative to Cyt *c* is able to break the heme ring [18]. It was earlier revealed that, under the influence of H_2O_2 on Cyt *c* (Fe^{3+}), the rate of Fe···S(Met80) bond breaking estimated by absorption at 695 nm was much greater (5 to 9 times) than that of porphyrin ring degradation (absorption in the Soret band, i.e., at 406 nm) [15]. Under the influence of a small (2- to 5-fold) excess of hydrogen peroxide, the level of A_{695} reached a plateau and, thus, a relatively stable product could be obtained—modified Cyt *c* having partly broken Fe···S(Met80) bond. As seen from Table 2, this partly modified Cyt *c* possesses a high peroxidase activity (2.5 times higher than that of the initial Cyt *c*). Nevertheless, the activating action on the chemical destruction of the Fe···S(Met80) bond cannot be compared with the action of forming a complex with cardiolipin. In the experiments with liposomes, no decrease in absorption at 695 nm was observed at a TOCL/PC/Cyt *c* ratio of 5 : 5 : 1, and the peroxidase activity became almost 8 times higher (Table 2). In other words, breaking of the Fe···S(Met80) bond causes only a small enhancement of Cyt *c* peroxidase activity, and binding of this hemoprotein with cardiolipin enhances greatly its peroxidase activity in the absence of visible changes in this bond.

DISCUSSION

It is the authors' opinion that the main result obtained in this work is that cardiolipin makes a specific activating effect on Cyt *c* peroxidase activity and it is

Table 2. Activation of cytochrome *c* (Cyt *c*) peroxidase activity on Fe···S(Met80) bond breaking and in the complex with cardiolipin-containing liposomes

Parameter	Cyt *c*	Oxidized Cyt *c*	Cyt *c* + PC–TOCL (1 : 5 : 5)*
EPR signal amplitude**	99 ± 7	301 ± 23	752 ± 54
A_{695}	$(10.8 \pm 0.1) \cdot 10^{-3}$	$(10.9 \pm 0.1) \cdot 10^{-3}$	$(10.9 \pm 0.1) \cdot 10^{-3}$

* Liposomes prepared from phosphatidylcholine and cardiolipin by the method described in [15] were used.
** The method of peroxidase activity estimation is described in [15].

much more powerful than the total effect on protein conformation and concomitant breaking of the coordinate bond of heme iron (Fe^{3+}) with methionine-80 residue sulfur (Met80). This conclusion is based on the comparison of the effect of cardiolipins (bovine heart cardiolipin and synthetic tetraoleyl cardiolipin) with the effect of nonspecific anionic detergent SDS. In a sense, the two ligands produce a similar effect on Cyt *c*: recharging cytochrome, they change its conformation which results in breaking of the coordinate Fe···S(Met80) bond. Changes in the protein conformation were also observed due to the appearance of Cyt *c* tryptophan fluorescence as the concentration of SDS (unpublished data) and TOCL [15] was increased. A good parallelism between the extent of A_{695} band discoloration and fluorescence intensity at 330-350 nm was also observed. It is shown that both the range of concentrations and the shape of the relationship between concentrations and changes in the absorption values at 695-700 nm are very similar with these two amphiphilic anions. It is of particular interest that the activation of Cyt *c* peroxidase activity in both cases occurs in strict accordance with the bond breaking. This means that the fact of bond breaking is necessary for Cyt *c* peroxidase activity to arise. But there the similarity of cardiolipin to SDS ends.

A radical difference in their action consists in an incomparable efficiency of their activation of peroxidase activity. At a low ligand/protein ratio of 4 : 1 and lower (i.e., at a ratio insufficient for total neutralization of eight positive charges on the surface of the Cyt *c* protein globule [15]), SDS manifests no recordable activation of cytochrome peroxidase activity while TOCL as well as BCL demonstrates a pronounced activating effect (Figs. 3 and 4, insert): an 8- and 14-fold enhancement of peroxidase activity at BCL/Cyt *c* molar ratios of 1 : 1 and 2 : 1, respectively. In other words, the first cardiolipin molecules bound by Cyt *c* cause activation of peroxidase activity which can not be explained by mere breaking of the bond between heme iron and the methionine residue sulfur.

Similar data were obtained in the experiments on breaking the Fe···S(Met80) bond by hydrogen peroxide. In itself a 70% breaking of the bond resulted in only a threefold enhancement of peroxidase activity, while the binding of cardiolipin with cardiolipin-containing liposomes in the same experiments brought about an 8-fold enhancement of peroxidase activity (Table 2), and no changes in A_{695} absorption band were observed within the experiment error, i.e., less than ± 4% of amplitude (data not shown). All these data suggest that breaking of the Fe···S(Met80) bond is not the only or even the main cause of the activation of Cyt *c* peroxidase activity by cardiolipin: the main role belongs to a specific rearrangement of the protein molecule which is a unique characteristic of cardiolipin.

A great difference between the activating effect of cardiolipin and that of SDS within the whole concentration range (0 to 50, mol/mol) in which A_{695} band decreases can in principle be due not only to the additional activating effect of cardiolipin but, on the contrary, to the inhibiting activity of SDS. This can be due, for instance, to the replacement of Met80 by Lys79 causing the disappearance of A_{695} band while heme iron stays in the hexacoordinated state. This evidently does not takes place with cardiolipin. Further great enhancement of Cyt *c* peroxidase activity occurs at higher SDS concentrations (over 200 molecules per Cyt *c* molecule); as the result, it becomes approximately equal to the maximal activity of the complex of this hemoprotein with cardiolipin (compare Figs. 6 and 3b) which is, nevertheless, already reached at a lipid/protein ratio of 50 : 1 and does not enhance thereafter (see also [15]). Only special investigations can reveal whether this effect can be associated with the transition of heme iron to the penta-coordination state or with a facilitated access to heme iron for hydrogen peroxide dissolved in the surrounding aqueous medium. Anyway, cardiolipin unlike the nonspecific anionic detergent makes changes in the conformation of Cyt *c* necessary for its maximal peroxidase activity already at relatively low concentrations.

Cardiolipin specific action on the behavior of protein electron carriers and on the functions of Cyt *c* has been noted by many authors [9, 11, 19-26]. The specific action of cardiolipin is mostly due to its much tighter binding to the surface of the cardiolipin-containing lipid layer, as compared to the lipid layer containing other anionic phospholipids such as phosphatidyl inositol or phosphatidyl serine [27, 28]. Binding of Cyt *c* to membranes has been investigated by many means including measuring the efficiency of energy transfer from fluorescently-labeled phospholipids to cytochrome heme [29].

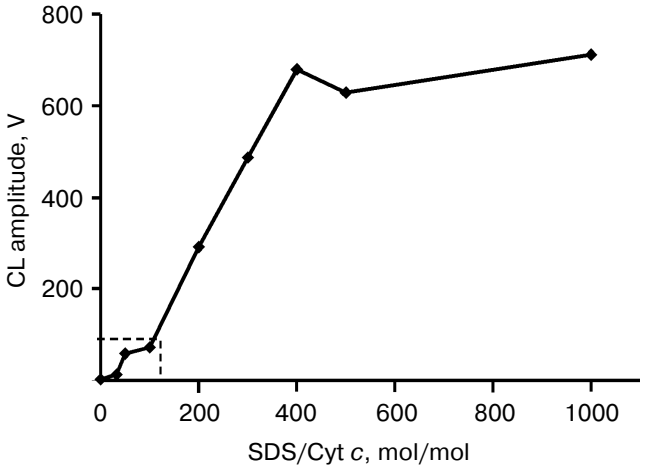

Fig. 6. Activation of Cyt *c* peroxidase activity at high SDS concentrations. Other conditions are the same as those in Fig. 3. The dashed line indicates the range of SDS/Cyt *c* concentrations < 100 : 1, corresponding to that shown in Fig. 3a.

The complex forms mainly due to electrostatic interactions though hydrophobic interactions contribute to the process as well [29].

Cytochrome also forms electrostatic complexes with other acidic lipids both in solutions and on the surface of liposome membranes; these complexes dissociate in the presence of salts or at millimolar ATP concentrations [30]. These and some other data [31] suggest that Cyt *c* contains two phospholipid-binding centers: the anion-binding center (A-center) and protonated-phospholipid-binding center (C-center) [30]. According to the scheme set out in work [32], a certain geometry of the phosphate group is necessary for cytochrome binding to the lipid bilayer surface, as well as the presence of two acyl chains, one of which is incorporated into the lipid bilayer and the other into the hydrophobic cavity inside the protein molecule [31]. Based on the data of the X-ray structure analysis of Cyt *c* crystals, it was assumed that protonated phosphate binds to asparagine residue Asn52 (horse heart Cyt *c*) while the phospholipid deprotonated polar group binds to lysine residues Lys72 and Lys73 [32]. The scheme suggested by Rytomaa was directly verified by experiments on quenching of the fluorescence of Cyt *c* zinc-substituted heme by bromated acyl lipid chains [33]; these results testify to a close contact between a certain chain section and heme.

Binding with cardiolipin changes the properties of the Cyt *c* molecule. Moreover, according to the data obtained by EPR [34], combinational scattering spectroscopy [35], and ^{31}P-NMR [36], the heme slot in Cyt *c* structure opens and heme iron changes the six-coordinate low spin state for the five-coordinate high spin state. D-NMR data also show that the polypeptide chain conformation changes and α-helices become destabilized [36]. It was shown by infrared spectroscopy that probably about 10% of α-helices get untwisted [37]. Results of NMR suggested that, on binding of Cyt *c* with cardiolipin, Met80 moves away from the heme, and the environment of Met65, which is part of a short α-helix segment not far from heme, changes [38].

All these specific changes must in some way promote an accelerated rate of the reaction between Cyt *c* heme and hydrogen peroxide, which is the first link in the enzyme peroxidase cycle. The mechanism of specific activation by cardiolipin of Cyt *c* peroxidase activity can be supposed to consist exactly in the facilitation of heme access for H_2O_2. Our next publication will present data supporting this supposition.

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