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Cardiolipin Activates Cytochrome *c* Peroxidase Activity Since It Facilitates H_2O_2 Access to Heme

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Abstract—In this work, the effect of liposomes consisting of tetraoleyl cardiolipin and dioleyl phosphatidylcholine (1 : 1, mol/mol) on the rate of three more reactions of Cyt *c* heme with H_2O_2 was studied: (i) Cyt *c* (Fe^{2+}) oxidation to Cyt *c* (Fe^{3+}), (ii) Fe···S(Met80) bond breaking, and (iii) heme porphyrin ring decomposition. It was revealed that the rates of all those reactions increased greatly in the presence of liposomes containing cardiolipin and not of those consisting of only phosphatidylcholine, and approximately to the same extent as peroxidase activity. These data suggest that cardiolipin activates specifically Cyt *c* peroxidase activity not only because it promotes Fe···S(Met80) bond breaking but also facilitates H_2O_2 penetration to the reaction center.

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Cytochrome *c* (Cyt *c*) is a carrier of electrons in mitochondria between respiratory complexes III and IV. Part of Cyt *c* is dissolved in the intermembranous space, though under certain conditions it can leave mitochondria, thus initiating a cascade of the reactions of programmed cell death, or apoptosis [1-4]. V. P. Skulachev et al. discovered a third function of cytochrome: oxidizing superoxide radical, ferrocytochrome *c* can play the role of antioxidant [2, 5]. Some recently published data show that a considerable part of Cyt *c* in mitochondria is rather tightly bound to cardiolipin, which is part of the inner mitochondrial membrane [6-14], and performs the fourth function of this hemoprotein, the function of peroxidase, which can play an important role in the initiation of Cyt *c* release from mitochondria and initiation of apoptosis [14, 15]. The mechanism of the activating effect of cardiolipin remains unrevealed, though some data exist suggesting that a significant role in this process belongs to changes in cytochrome conformation [6-8, 12, 16] which result in the breaking of the bond between heme iron and Met80 sulfur [17, 18].

The mechanism of action of Cyt *c* peroxidase is similar to that of other peroxidases, such as horseradish peroxidase, myeloperoxidase, and microperoxidase. Using the stopped-flow method, scanning spectrophotometry, and EPR [19-21], and cooling enzymes to −40°C in 50% methanol [22], the properties of intermediate products were studied for myeloperoxidases 8 and 11, and the rate constants for basic stages were determined [21]. Formation of protein radical (Compound I) characteristic of all peroxidases was also revealed in the process of interaction of H_2O_2 with Cyt *c* by the method of EPR using spin traps [23, 24] and recording the singlet EPR signal at low temperature [14, 25, 26].

The experiments with microperoxidases revealed that the Michaelis constant in the reaction with organic substrate was different with microperoxidases having different peptide chain length (6, 8, and 11) at an equal

Abbreviations: BCL) bovine heart cardiolipin; CL) chemiluminescence in the presence of luminol; Cyt *c*) cytochrome *c*; DTPA) diethylenetriaminepentaacetate; PC) 1,2-dioleyl-*sn*-glycero-3-phosphocholine; TOCL) synthetic 1,1′,2,2′-tetraoleyl cardiolipin.

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maximal reaction rate [27]. Though the rate of product formation in the reaction of Compound II of microperoxidases with organic substrates differed with different substrates [19], a great enhancement of Cyt *c* peroxidase activity by cardiolipin is probably caused by the acceleration of the initial (and not final) stage of the reaction, in particular, interaction between cytochrome and H_2O_2 with the formation of Compound I. Under similar conditions, the effect of cardiolipin and other anionic lipids on Cyt *c* peroxidase activity and radical formation was approximately equal in magnitude [15, 28].

At least two origins of differences in the activity of free and cardiolipin-bound Cyt *c* can exist: (i) Fe···S(Met80) bond breaking, and (ii) changes in heme microenvironment which facilitates the diffusion of H_2O_2 to the reaction center. In classical peroxidases, such as horseradish peroxidase and myeloperoxidase, the heme iron atom exists in the pentacoordinate form, and H_2O_2 is thus able to bind by the sixth coordination bond. These hemoproteins demonstrate peroxidase activity several orders of magnitude greater than that of Cyt *c*, whose sixth coordination bond Fe^{3+} is occupied by Met80. Partial proteolysis of Cyt *c* results in the formation of microperoxidases in which the Fe···S(Met80) bond is absent and the peroxidase activity is greater than that of Cyt *c* by three orders of magnitude. Peroxidase activity was enhanced under chemical actions on Cyt *c* that broke the Fe···S(Met80) bond, such as carboxymethylation [29] and the action of peroxynitrite [30] or hypochlorite [31]. Under the influence of anionic lipids including cardiolipin, the Cyt *c* globule surface recharges and unfolds, Fe···S(Met80) bond breaks, and Cyt *c* peroxidase activity is enhanced [28].

In our previous work, a quantitative parallelism was demonstrated between the enhancement of peroxidase activity and the disappearance of the absorption band A_{695} arising from the Fe···S(Met80) bond. Nevertheless, the very fact of Fe···S(Met80) bond breaking can not completely account for cardiolipin activating effect on Cyt *c* peroxidase activity since, in spite of the same effect on the bond, the activating effect on the peroxidase activity was much greater with cardiolipin than with the anionic surfactant dodecyl sulfate, and Fe···S(Met80) bond breaking under the influence of H_2O_2 had several times smaller effect than formation of a complex with cardiolipin under the conditions when Fe···S(Met80) was practically unaffected by the lipid [28].

In spite of a number of publications about differences in the electrochemical characteristics of myeloperoxidases and Cyt *c* [32-36], the role of changing of the heme redox potential in cardiolipin activation of Cyt *c* peroxidase activity remains unclear.

Change in the heme microenvironment that facilitates H_2O_2 diffusion to the reaction center can be one more cause of the enhanced peroxidase activity of Cyt *c* on the formation of its complex with cardiolipin. Under the influence of denaturants, such as guanidine or carbamide [37], and anionic lipids [38, 39], a partial unfolding of Cyt *c* globule occurs. When treated with maleic anhydride, Cyt *c* undergoes conformational changes [40] which results in Fe···S(Met80) bond breaking and heme redox potential alteration [41]. In all studied instances, changes in heme spin state occurred, the absorption disappeared at 695 nm, and changes in the circular dichroism in the Soret band (at 416 nm) were observed [42]. All these data suggest a close parallelism between the changing of protein conformation caused by perturbing agent and the existence of the Fe···S(Met80) bond, which is not always observed [37]. This circumstance makes it very difficult to answer the question whether Cyt *c* conformation change caused by cardiolipin is confined to only Fe···S(Met80) bond breaking.

In the course of this research, data were obtained proving the hypothesis that cardiolipin is able to facilitate H_2O_2 penetration to the active center of Cyt *c*. It was revealed that not only peroxidase activity enhanced under the influence of cardiolipin, but also three more reactions of H_2O_2 heme accelerated: (i) oxidation of Cyt *c* (Fe^{2+}) to Cyt *c* (Fe^{3+}), (ii) breaking of Fe···S(Met80) bond, and (iii) decomposition of the system of conjugated bonds in the heme porphyrin ring. The acceleration of all those reactions was activated by cardiolipin to the same extent as peroxidase activity was.

MATERIALS AND METHODS

Reagents. Horse heart Cyt *c* (99% purity) was obtained from Sigma (USA). The concentration was determined spectrophotometrically after reduction by 99% ascorbic acid (Aldrich, USA) using the molar absorptivity at the maximum of the Soret band (550 nm) ε_{550} = 29,400 liter/mole·cm. Bovine heart cardiolipin (BCL) (98% purity) and ascorbate oxidase were obtained from Sigma; 1,1′,2,2′-tetraoleyl cardiolipin (sodium salt) (TOCL) and 1,2-dioleyl-*sn*-glycero-3-phosphocholine (PC) were from Avanti (USA); 97% diethylenetriaminepentaacetate (DTPA) was from Aldrich.

Hydrogen peroxide was prepared by dilution from 30% H_2O_2 (Aldrich). Hydrogen peroxide concentration was determined spectrophotometrically using ε_{230} = 72.1 liter/mole·cm [43]. $NaH_2PO_4 \cdot 2H_2O$ (analytical grade; Khimmed, Russia) was used to prepare a phosphate buffer solution (PB), pH 7.4.

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). Liposomes were added to a Cyt *c* solution to obtain a necessary lipid/protein molar ratio.

The initial solutions of Cyt *c*, cardiolipin, TOCL, and a 20 mM phosphate buffer solution were prepared using deionized water. The total volume of the sample was 1 ml.

Cytochrome *c* absorption spectra and kinetics of reactions were investigated at room temperature using a Shimadzu spectrophotometer UV160U (Japan). The absorption of Cyt *c* complexes by liposomes was measured against a suspension of liposomes containing no cytochrome. To reduce the effect of light scattering, the cuvettes were placed as close as possible to the measuring block's windows. The kinetics of ascorbic acid reduction of Cyt *c* was measured by recording of a series of spectra at a recording interval of 1 min after the addition of 100 μM ascorbate to a sample containing 40 μM Cyt *c*. After the ferrocytochrome *c* accumulation curve reached the plateau in the experiments on hydrogen peroxide oxidation of ferrocytochrome *c*, ascorbate oxidase (2 μl) was added to the sample at a concentration sufficient for decomposition of all the ascorbate within 1 sec; 200 μM H_2O_2 was then added, and a successive series of spectra was recorded at 1 min intervals until the spectrum stopped changing. The relationship of $A_{549}-A_{530}$ versus time was calculated. The sample exchange technique was applied in a number of cases during the experiments on the recording of cytochrome weak absorption band at 695-700 nm against a background of a high light scattering and a decrease in basic absorption bands lying in the shorter-wavelength region. Two cuvettes containing a suspension of liposomes (1) without Cyt *c* and (2) with Cyt *c* were placed into the spectrophotometer; cuvette (2) served as the comparison sample, and cuvette (1) as the measurement sample. After setting and recording zero line, the samples were swapped, and the absorption spectrum was recorded. Such spectra had a double amplitude as compared to standard absorption spectra and allowed obtaining more precise shape and amplitude of weak absorption bands, such as the band of Fe···S(Met80) in the region of 700 nm. In the course of spectrophotometry data processing, the background signal of shorter-wavelength bands was subtracted from the optical density; that signal decreased in the region of 670-730 nm approximately by the linear law, as shown by special measurements. The amplitude of such a calculated spectrum or the sum of the optical densities in all the band was used to estimate the amount of unbroken Fe···S(Met80) bonds. In the experiments on hydrogen peroxide breaking of those bonds, 2000 μM H_2O_2 (50-fold excess in relation to Cyt *c*) was added to the samples containing 40 μM Cyt *c*, and a decrease in absorption in the band of 670-730 nm was measured as described.

RESULTS

It was shown in previous research that Fe···S(Met80) bond breaking in Cyt *c* was not the only cause of a drastic activation of the peroxidase activity of the cytochrome on its binding with cardiolipin [28, 44] since, at the same extent of bond breaking, cardiolipin activated cytochrome peroxidase activity much more intensively than the anionic surfactant dodecyl sulfate; and on the chemical breakage of the bond, the enhancement of cytochrome peroxidase activity was incomparably less than that under the influence of cardiolipin-containing liposomes in the amounts when the absorption of the bond at 695 nm practically did not change [44]. It was assumed that cardiolipin, binding with the Cyt *c* globule, alters specifically its conformation in such a way that it makes the penetration of water-dissolved H_2O_2 to the hemoprotein active center much easier. To test this hypothesis, three other reactions between H_2O_2 and Cyt *c* heme group were studied: (i) ferrocytochrome *c* oxidation to ferricytochrome *c*, (ii) Fe···S(Met80) bond breaking, and (iii) porphyrin ring degradation under the influence of high H_2O_2 concentrations.

Oxidation of Cyt *c* (Fe^{2+}) by hydrogen peroxide. The absorption spectra of ferro- and ferricytochrome *c* in the mixture with liposomes did not differ from the spectra of the solution of only Cyt *c* in a phosphate buffer solution.

In our experiments, the effect of cardiolipin on hydrogen peroxide oxidation of Cyt *c* (Fe^{2+}) has been investigated. A cytochrome *c* solution or a suspension containing Cyt *c* and liposomes consisting of BCL and PC at molar ratios of 1 : 5 : 5 or 1 : 0 : 10 were first subjected to reduction with ascorbic acid in stoichiometric amounts, and then oxidized under the influence of H_2O_2 at a concentration 50-fold exceeding that of Cyt *c* (see "Materials and Methods"). Spectral measurements

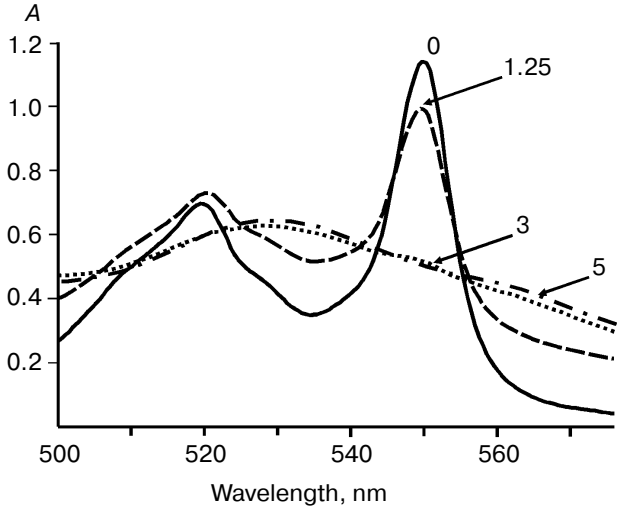

Fig. 1. Changes in Cyt *c* (Fe^{2+}) absorption spectrum under the influence of 200 μM H_2O_2. The figures by the curves designate the time after H_2O_2 addition (in minutes). Final concentrations: 40 μM Cyt *c*, 400 μM TOCL and PC (each), 100 μM ascorbate, 20 mM phosphate buffer, pH 7.4.

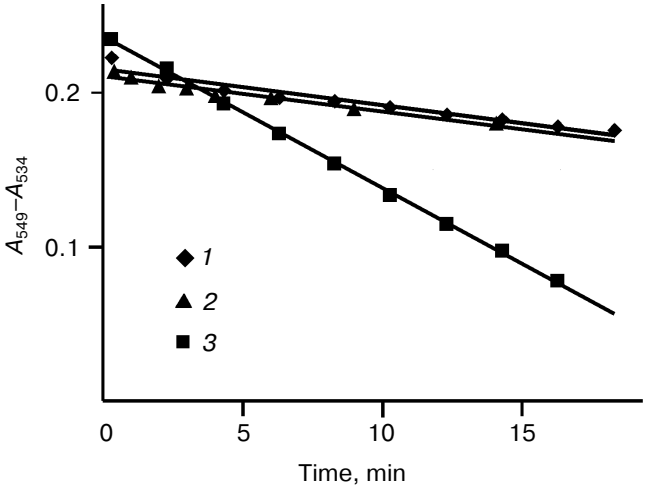

Fig. 2. Kinetic curves of ferrocytochrome *c* oxidation: *1*) Cyt *c* ($y = -0.0023x + 0.2158$); *2*) Cyt *c* + PC (1 : 10) ($y = -0.0023x + 0.2114$); *3*) Cyt *c* + TOCL + PC (1 : 5 : 5) ($y = -0.0099x + 0.2369$). The curves are constructed by experimental points with the least-squares technique. A 40-μM Cyt *c* was added to a suspension of liposomes formed from tetraoleyl cardiolipin and dioleyl phosphatidylcholine (final concentrations 400 μM each). After 30 min of incubation, ascorbate was introduced to a concentration of 100 μM and the reduction kinetics were monitored by changes in the absorption spectra. After the spectra stopped changing, 2 μl of ascorbate oxidase and 200 μM H_2O_2 were added. The spectra were scanned every other minute, and the kinetics of heme oxidation was followed by the changing difference of optical densities between 549 and 534 nm.

showed that the peroxide transformed ferrocytochrome to ferricytochrome, and other light-absorbing products were not noted (Fig. 1). In all cases, ferrocytochrome oxidation had a constant rate; the rate was the same with free cytochrome and Cyt *c* in the presence of liposomes from phosphatidylcholine (Cyt *c*/PC = 1 : 10), and increased by 4.3 times in the presence of liposomes containing cardiolipin (Cyt *c*/BCL/PC = 1 : 5 : 5) (Fig. 2). The same was observed at higher phospholipids/cytochrome ratios, though the activating effect of cardiolipin was more pronounced (table).

Fe···S(Met80) bond breaking under the influence of H_2O_2. No Fe···S(Met80) bond breaking, i.e., noticeable change in the absorption band of 695-700 nm (spectra not shown), was observed at hydrogen peroxide concentrations below 200 μM used in the experiments on peroxidase activity determination and in those on Cyt *c* (Fe^{2+}) oxidation to Cyt *c* (Fe^{3+}) where the H_2O_2/Cyt *c* ratio did not exceed 5 : 1. Nevertheless, at millimolar H_2O_2 concentrations, the bond breaking occurred (Fig. 3b), as well as a much lower degradation of the system of conjugated bonds responsible for the absorption within the Soret band (Fig. 3a and [20]) and at 530 nm (Fig. 3b). It was of interest to find out in what way the reactions between H_2O_2 and heme depended on the binding of Cyt *c* with cardiolipin in lipid membranes.

Figure 4 shows change in absorption of the Fe···S(Met80) bond in a cytochrome solution (Fig. 4, curve *1*) and in the mixture of Cyt *c* with cardiolipin-containing liposomes (Fig. 4, curve *2*). It is not difficult to note a startling difference between the rates of these reactions: the initial slope of the curves differed 10-fold (9.8 ± 2.5) with liposomes at a Cyt *c*/BCL/PC ratio of 1 : 10 : 10; while liposomes containing phosphatidylcholine alone (Cyt *c*/BCL/PC = 1 : 0 : 20) had no significant effect on the bond breakage at 695-700 nm under the influence of H_2O_2 (the data not shown). When measuring the absorption within the Soret band, it was revealed that cardiolipin also accelerated heme degradation under the influence of hydrogen peroxide.

How would the Fe···S(Met80) coordination bond behave if H_2O_2 is added not to oxidized but to reduced Cyt *c*? The answer to this question can be found in Fig. 5. At first, when Cyt *c* (Fe^{2+}) is oxidized to Cyt *c* (Fe^{3+}), the absorption band appears at 695 nm, which is absent in ferrocytochrome (the beginning of the curve in Fig. 5b), and then the Fe···S(Met80) bond breaks (the sloping segment of the absorption curve $A_{680\text{-}720}$). As already mentioned, both reactions are accelerated by cardiolipin.

DISCUSSION

Thus, the binding of Cyt *c* with cardiolipin on the surface of the lipid bilayer of membranes accelerates not only the peroxidation of organic compounds, as shown by EPR, fluorescence, and chemiluminescence [19, 20], but other reactions of H_2O_2 with heme: oxidation of Cyt *c* (Fe^{2+}), and breaking of Fe···S(Met80) bond and the system of conjugated double bonds in the porphyrin ring (a slower reaction). This circumstance seems to be of con-

Rate of hydrogen peroxide oxidation of ferrocytochrome *c* in solution and liposome suspension

Cyt *c*/TOCL/PC molar ratio	Linear approximation reliability, R^2	Oxidation rate, %
1 : 0 : 0	0.94	100
1 : 4 : 4	1.00	377
1 : 0 : 8	0.96	97.6
1 : 0 : 0	0.97	100
1 : 5 : 5	1.00	430
1 : 0 : 10	0.96	100
1 : 0 : 0	0.95	100
1 : 10 : 10	1.00	1983
1 : 0 : 20	0.96	100

siderable importance for understanding the mechanism of activation of Cyt *c* peroxidase activity on its binding with cardiolipin. The apparent reason for the low peroxidase activity of Cyt *c* is that all six coordination bonds of the catalytically active atom, heme trivalent iron, in this protein are occupied, unlike in genuine peroxidases such as myeloperoxidase or horseradish peroxidase where one bond is free, and it is the bond by which the primary peroxidase substrate, H_2O_2, attaches [21]. In the presence of negatively charged lipids including cardiolipin, the protein globule partially unfolds, the Fe···S(Met80) bond breaks, and the activation of Cyt *c* peroxidase activity follows [22, 23]. Such a viewpoint (see [14, 15]) is corroborated by the fact that the Fe···S(Met80) bond breaking, for instance, under the influence of hydrogen peroxide [19, 20], or hypochlorite [24], or on carbomethylation [25], results in the enhancement of Cyt *c* peroxidase activity. The data obtained in our two latest works and in the present research suggest that the scheme *anionic lipid action → Cyt c surface recharging → globule unfolding → Fe···S(Me-80) bond breaking → Cyt c peroxidase activity* is not, however, able to explain all the experimental data, especially relating not to the too-far-gone changes in the

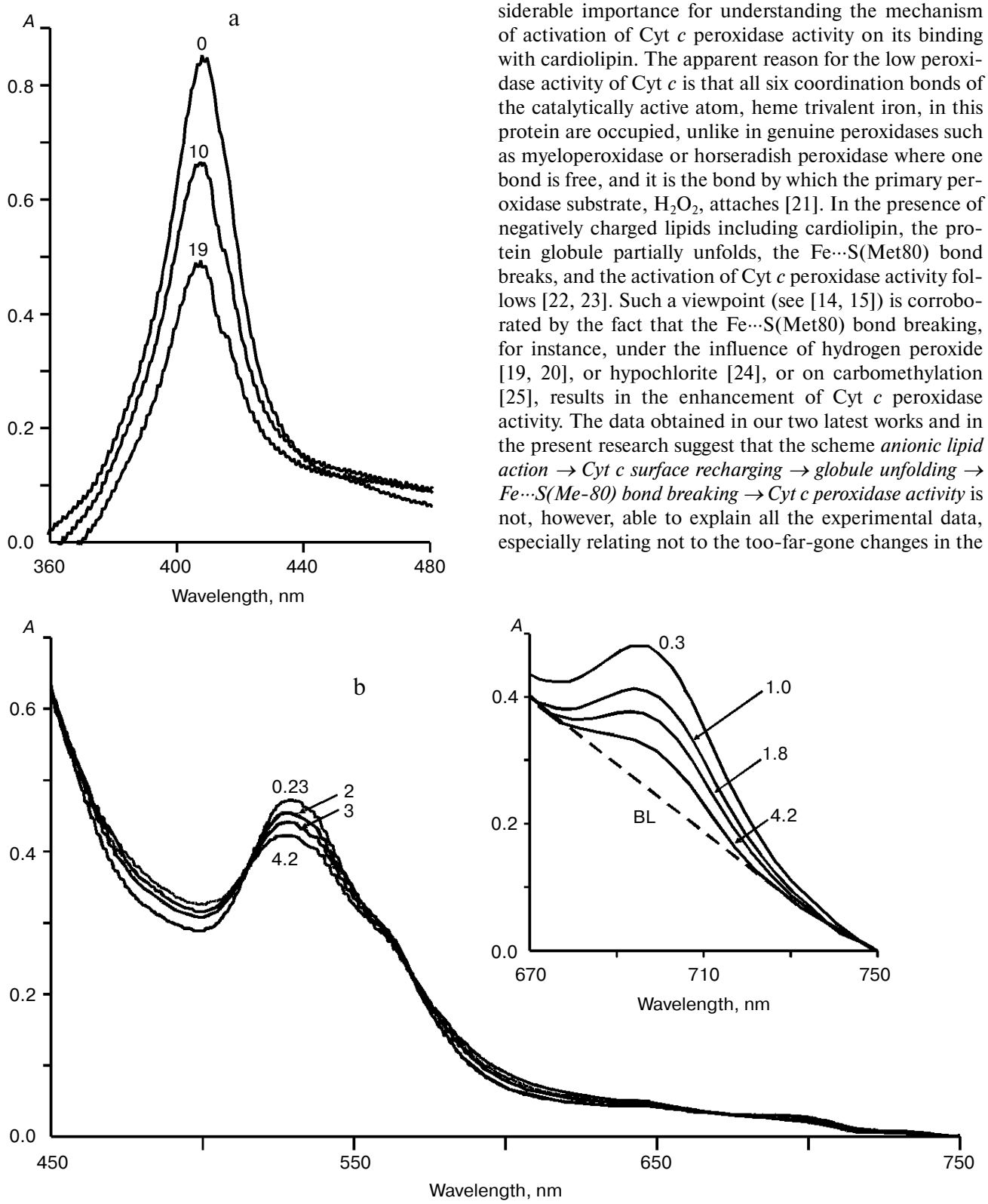

Fig. 3. Changes in Cyt *c* absorption spectra under the influence of H_2O_2. a) Change of absorption in the Soret band; b) that in the long-wavelength region of the spectrum; insert, absorption in the region of 670 to 750 nm imputed to the Fe···S(Met80) coordination bond. The figures by the curves designate the start of scanning of the spectra after the moment of addition of 1.025 mM H_2O_2 to a Cyt *c* solution. BL, a base line subtracted from the spectra for plotting of optical density of the absorption of Fe···S(Met80) against the time after H_2O_2 addition.

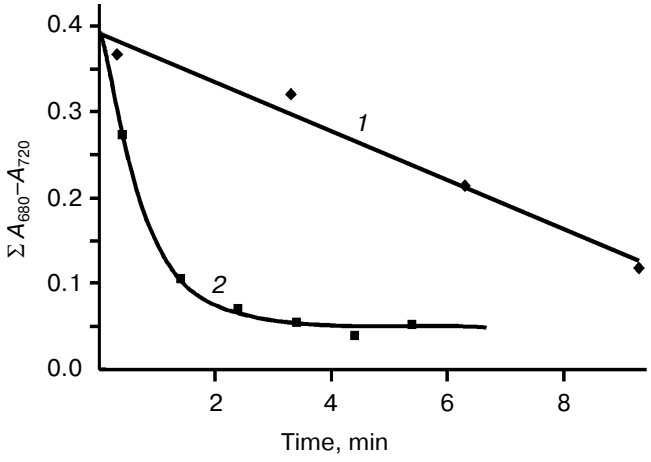

Fig. 4. Effect of cardiolipin on the rate of hydrogen peroxide-induced destruction of the Fe···S(Met80) bond. The kinetics of changing absorption in the region of 680-720 nm in a Cyt *c* solution (Fe^{3+}) (*1*) and in the mixture of Cyt *c* with liposomes consisting of TOCL and PC (*2*). Time – the start of spectra scanning. H_2O_2 (2 mM) was added at the initial moment of time. Cyt *c*/TOCL/PC molar ratios are 1 : 10 : 10. The area between the optical density curves and the baseline is laid off along the ordinate (Fig. 3b, insert). The area was calculated as the sum of optical densities within the range of 680-720 nm wavelengths at a count length of 0.2 nm. The incubation mixture contained 40 μM Cyt *c* (Fe^{3+}), liposomes at a final concentration of BCL and PC of 400 μM each. The original slopes differ by (9.8 ± 2.5)-fold.

protein structure at high (many dozens) lipid/protein ratios, but under milder conditions, i.e., at a ligand/protein ratio of not very much exceeding 10 : 1. Two facts revealed in our previous research seem to be of most importance [20]:

– the nonspecific anionic surfactant SDS, at a ligand/protein ratio below 50 : 1 (at which all Fe···S(Met80) bonds break), activated Cyt *c* peroxidase activity by one-two orders of magnitude more weakly at the same extent of bond breaking;

– chemical breaking of 70% of the Fe···S(Met80) bonds by hydrogen peroxide resulted in only three-fold enhancement of Cyt *c* peroxidase activity, while 10-fold enhancement of this activity in the complexes of Cyt *c* with cardiolipin-containing liposomes was not accompanied by a measurable change in the absorption band at 695 nm characteristic of Fe···S(Met80).

These facts suggest that the effect of cardiolipin is *specific* and is not only confined to the protein surface recharging and the associated Fe···S(Met80) bond breaking. It is possible to think that this specific effect consists in the changing of the conformation of a definite segment of the protein molecule, which facilitates the access of hydrogen peroxide to the active center. It was shown in this work that other reactions of H_2O_2 with heme, i.e., oxidation of Cyt *c* (Fe^{2+}) to Cyt *c* (Fe^{3+}), Fe···S(Met80) bond breaking, and apparently tetrapyrrole ring decom-

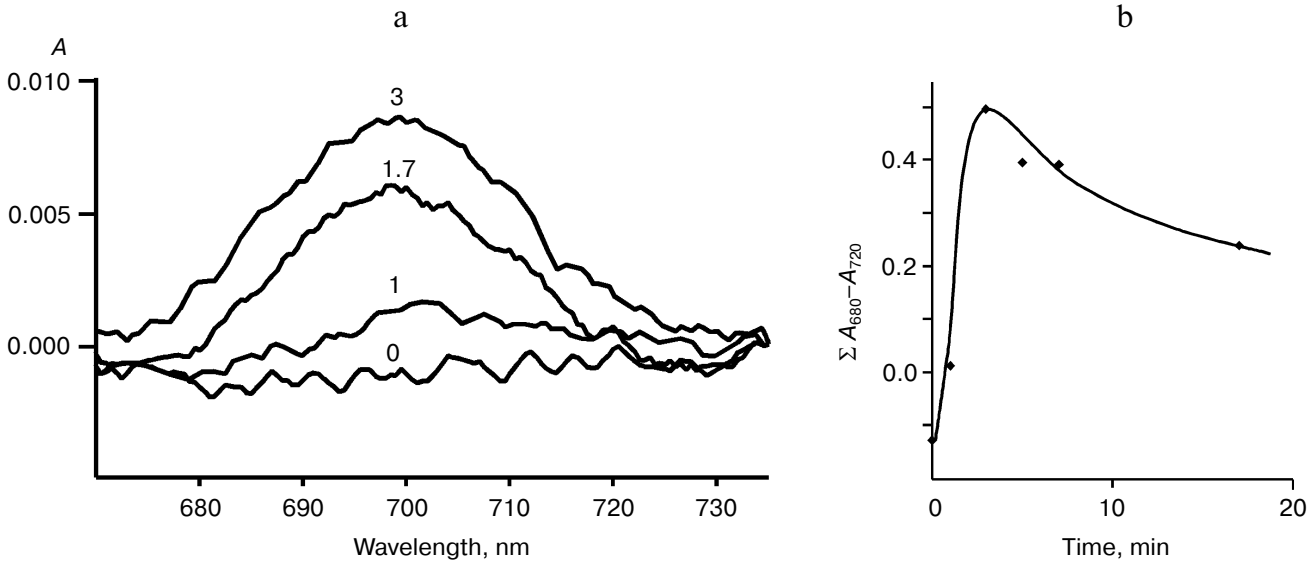

Fig. 5. Hydrogen peroxide-induced oxidation of Cyt *c* (Fe^{2+}). a) A_{695} band built up with the subtraction of baseline absorption between 670 and 735 nm (see Fig. 3b, insert, and "Materials and Methods"). The figures by the curves designate the time after H_2O_2 addition (in minutes). b) Changing of the area under the absorption curve in the region of 680-720 nm (wavelength intervals 0.2 nm) after the addition of H_2O_2. The incubation mixture contained 40 μM Cyt *c* (Fe^{3+}), liposomes at a final concentration of TOCL and PC of 400 μM each. Cyt *c* (Fe^{3+}) was reduced by ascorbate as shown in Fig. 3. Zero time on the curves corresponds to the moment of addition of H_2O_2 to final concentration of 200 μM.

position, were activated greatly on binding of Cyt *c* with lipid vesicles containing cardiolipin, and approximately to the same extent as the peroxidase activity was. A touch of the reacting molecule with the porphyrin ring edge would apparently suffice for a standard electron transfer reaction (*the extraspherical electron transfer mechanism*) (it is known that the heme ring edge in the Cyt *c* molecule is immersed into the water solution [26]), while H_2O_2 reactions need a direct contact of this molecule with heme iron hidden deep inside the protein globule (*the intraspherical electron transfer mechanism*). It cannot be excluded that this is an evolutionary accommodation aimed at the inhibition of an unauthorized decomposition of the protein and surrounding molecules by a reactive particle such as H_2O_2. Therefore, one more scheme of a drastic and *specific* enhancement of Cyt *c* peroxidase activity by cardiolipin can be suggested: *binding of cardiolipin→ changing of the conformation of a globule segment (different from that on which Fe···S(Met80) bond breaking depended) → facilitation of the access to the active center for H_2O_2 dissolved in the aqueous medium surrounding the protein → a drastic acceleration of hydrogen peroxide oxidation of organic substrates.*

These two mechanisms of action of cardiolipin most likely function jointly, though it is not clear whether the events occur simultaneously or successively. Moreover, it is necessary to find out what particular changes in the atomic structure of Cyt *c* underlie the cardiolipin-specific activation of the peroxidase activity of this hemoprotein.

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