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Structure of the complex of cytochrome *c* with cardiolipin in non-polar environment

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ABSTRACT

The complex of mitochondrial protein cytochrome *c* (CytC) with anionic phospholipid cardiolipin (CL) plays a crucial role in the initiation of apoptosis by catalyzing lipid peroxidation in mitochondrial membranes. In our previous papers, we found that CytC and CL mixed in millimolar concentrations form a sediment showing microcrystals composed of nanospheres (Cyt-CL) of 11–12 and 8 nm in diameter. The hypothesis was proposed that Cyt-CL, having hydrophobic shell, may appear inside the membrane lipid bilayer in mitochondria and peroxidase membrane phospholipids so initiating the apoptotic cascade. In this work, Cyt-CL complex dissolved in chloroform or hexane was investigated as a model of the complex in mitochondrial membranes. We used dynamic light scattering method to measure the size of the particles. The analysis of particles size distribution of Cyt-CL in chloroform allows to reveal three dominant diameters of 12.1 ± 1.4, 7.8 ± 1.0, and 4.7 ± 0.7 nm. The first two values are closed to those, earlier obtained with small-angle X-ray scattering method in Cyt-CL microcrystals, 11.1 ± 1.0 and 8.0 ± 0.7 nm. CL extracted in chloroform-methanol forms a real solution of particles with diameter of 0.7 ± 0.1 nm. In methanol-water phase, CL and CL + CytC mixture form particles of 83.7 ± 9.8 and 71.3 ± 11.6 nm, respectively. Apparently, cardiolipin in 50% methanol forms single-layer liposomes regardless of the presence of CytC in the medium. Partial unfolding of CytC in the complex was evidenced by (a) appearance of fluorescence of tyrosine and tryptophan residues and (b) disappearance of the absorption band at 699 nm due to breakdown of heme iron – methionine bond > F···S(Met80). In hydrophobic solvent Cyt-CL exhibited quasi-lipoperoxidase and lipoxygenase activity as was shown in kinetic measurements of chemiluminescence enhanced by coumarin C-525, a selective sensitizer of chemiluminescence, associated with reactions of lipid peroxyl radicals.

Our data in this model system do not contradict the hypothesis (Vladimirov, Y.A. et al. Biochemistry (Mosc) 78, 1086–1097) that nanospheres of Cyt-CL complex, embedded into the lipid phase of mitochondrial membrane, catalyze lipid peroxidation, thereby initiating apoptosis.

1. Introduction

1.1. The biological function of the complex of CytC with CL

Over the past decade, the biological role of the complex of CytC with CL formed in the lipid membranes of mitochondria has attracted significant interest from biologists and chemists. This, for instance, is indicated in the leading research performed in the laboratory of V.E. Kagan at the University of Pittsburgh (Kagan et al., 2009a, 2004, 2005; Tyurina et al., 2014). The role of the complex is associated with initiation of apoptosis (Kagan et al., 2009a, 2004), which is an indispensable process for development, aging and death of living organisms.

Abbreviations: CytC, cytochrome *c*; CL, cardiolipin; TOCL, 1,1′,2,2′-tetraoleylcardiolipin; BCL, cardiolipin from bovine heart; Cyt-CL, complex of cytochrome *c* with cardiolipin; SAXS, small-angle X-ray scattering; DLS, dynamic light scattering

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On the other hand, together with the well-known cytosolic enzymes such as cyclooxygenases and lipoxygenases, the Cyt-CL complex is directly involved into biosynthesis of extremely important metabolic regulators – lipid mediators (Tyurina et al., 2014).

The protein-lipid complex of CytC with CL has recently been the subject of a large number of studies, since the formation of the complex plays a key role in triggering the cascade of reactions in the cell leading to its programmed death, apoptosis (Kagan et al., 2004, 2005). This main function of Cyt-CL is realized in the lipid bilayer of the mitochondrial membrane. It is accompanied by oxidation of unsaturated fatty acid residues of phospholipids including, in the first place, CL. Eventually, Cyt-CL can reach the site of convergence of the outer and inner mitochondrial membranes, where lipid peroxidation damages the VDAC (Voltage Dependent Anion Channel)-ANT (adenine nucleotide translocase) complex. This in turn leads to reduction of the barrier property of the internal membrane, swelling of the matrix, appearance of large pores in the outer membrane, and finally to the release of CytC into cytoplasm that leads to launching of the apoptotic cascade (Skulachev, 1998). While, up to now the structure of the complex and the mechanism of its function are not well understood. Most of researchers support the hypothesis of membrane-bound CytC (Brown and Wuthrich, 1977) and the review (Kagan et al., 2009b). According to this hypothesis, CytC is attached to the surface of the lipid bilayer that results in changing the protein conformation (Brown and Wuthrich, 1977; Kobayashi et al., 2016; Rytomaa and Kinnunen, 1995; Snider et al., 2013; Spooner and Watts, 1991a,b) (Vladimirov et al., 2013b).

1.2. Cytochrome c forms with cardiolipin a complex in the form of nanospheres (Cyt-CL)

In our previous communication, the we showed that the complex of CytC with 1,1′,2,2′-tetraoleylcardiolipin (TOCL), prepared by mixing a concentrated solution of cytochrome *c* and TOCL dissolved in small volume of methanol, is poorly soluble in water and precipitates (Vladimirov et al., 2013a, 2011). The SAXS study of the sediment has demonstrated that the complex of CytC with TOCL consists of microcrystals showing interplanar reflection distances of 8.0 and 11.1 nm (Proskurnina et al., 2013; Shtykova et al., 2013; Vladimirov et al., 2013a, 2011). The spectrophotometric determination of Cyt-CL concentration changes in the supernatant, upon the addition of a given amounts of the cardiolipin, showed that the stoichiometric ratio CL/CytC is constant within a wide range of Cyt-CL concentrations and was about 35/1 at pH 7. These data allowed to assume that the complex Cyt-CL is a nanosphere of approximately 11.2 nm in diameter, formed by one CytC molecule and a monolayer of CL surrounding this molecule (Vladimirov et al., 2011). At low pH (3.5–5.5) the protein to lipid ratio in Cyt-CL was found to be essentially lower (11–12) than that at neutral medium and that the size of cells in the microcrystals can be equal to 11.1 ± 1.0 nm and/or 8.0 ± 0.7 nm; with the contribution of these two structures depending on pH (Proskurnina et al., 2013; Shtykova et al., 2013; Vladimirov et al., 2013a).

1.3. Partial unfolding of CytC in Cyt-CL nanoparticles

Some reports support the hypothesis that the structure of CytC in the Cyt-CL complex differs from that of native CytC (Vladimirov et al., 2013b). The work Choi and Swanson (1995) reports an influence of bovine cardiolipin and dioleoylphosphatidylglycerol on the structure of CytC at the protein:lipid ratio of 1:100. The number of alpha helices decreased by 10 and 5% and the number of beta-structures increased by 15 and 5% in the presence of bovine cardiolipin and dioleoylphosphatidylglycerol, respectively. Other authors have shown that the interaction of CytC with CL-containing phospholipid membrane results in partial unfolding of the protein and dissociation of the Met80 from the heme (Belikova et al., 2006; Kapralov et al., 2007; Mandal et al., 2015; O'Brien et al., 2015; Patriarca et al., 2009; Sinibaldi et al., 2008). A partial unfolding of the CytC macromolecule was supposed to occur also in the nanospheres of Cyt-CL sediment (Vladimirov et al., 2011). Considering the fact that the diameter of the nanosphere is 11.2 nm and the thickness of two layers of TOCL is approximately 5.6 nm, the size of the CytC globule should be estimated as approximately 5.6 nm, which significantly exceeds the size of the globule of native CytC (3.5 nm × 3.9 nm, as found in the protein database for the structure of PDB IP 3NBS and our SAXS results (Vladimirov et al., 2011)). This means that the formation of the Cyt-CL complex leads to a remarkable increase in the dimensions of the CytC molecule inducing its partial melting.

The increase in size of the CytC molecule in the complex with CL is confirmed by analysis of fluorescence of the complex. The addition of CytC to CL leads to appearance of fluorescence of the tyrosine (Tyr) and tryptophan (Trp) residues, which is not observed in native cytochrome (Belikova et al., 2006; Kapralov et al., 2011) due to energy transfer from Tyr or Trp to the heme located nearby. The appearance of fluorescence is caused by an increase in the distance of the residues of Tyr and Trp from the heme due to partial melting of the CytC globule.

1.4. Peroxidase activity of Cyt-CL

In previous studies, we showed that formation of Cyt-CL complex in water leads to the rupture of iron-sulfur bond between heme iron and methionine Met-80 (Belikova et al., 2006; Vladimirov et al., 2011). This bond is absent in other hemoproteins, which have peroxidase activity. The native CytC has this bond that prevents binding of the heme with H_2O_2 and therefore the native CytC does not possess the peroxidase activity. It is important to note that this bond has a characteristic absorption peak in the region of 700 nm, which makes it possible to probe the presence of the bond spectrophotometrically. It was shown that when Cyt-CL complex is formed, the structure of an active center of CytC changes, which allows H_2O_2 to penetrate the CytC active site (Vladimirov et al., 2006b) and, as a result, facilitates the appearance of peroxidase activity (Belikova et al., 2006). Studying the rupture of iron-methionine bonds in CytC by monitoring its absorption at 700 nm has revealed a correlation between the extent of the bond breakage and the level of peroxidase activity (Vladimirov et al., 2006c).

The hydrophobic nanoparticles of Cyt-CL perform lipid peroxidation in mitochondrial membranes, most likely directly in the interior of the inner membrane being embedded between the two lipid monolayers forming the lipid bilayer (Vladimirov et al., 2013b). Recently, we have demonstrated that the Cyt-CL complex can be formed in non-polar solvents such as chloroform and hexane (Vikulina et al., 2015) and the complex can catalyze formation of lipid radicals in both quasi-lipoxygenase and lipoperoxidase reactions (Vladimirov et al., 2017).

1.5. The purpose of this study

Self-assembly of Cyt-CL nanoparticles in non-polar environment presents an easy and elegant approach for the mimics of Cyt-CL embedded into the membrane by surrounding of CytC-CL complex by lipophilic phase, e.g. straight-chained saturated hydrocarbons. However, to the best of our knowledge, there is no literature on the structure of the Cyt-CL complex in non-polar environment. The structure of Cyt-CL complex in non-polar environment cannot be deduced from crystal phase and direct evidence of the complexation and analyses would shed light on the complex structure and its vital role in apoptosis. Therefore, the purpose of this study was to investigate the structure of the Cyt-CL complex in non-polar environment through analysis of the size, absorbance, and fluorescence of the complex and to probe its enzymatic activity by chemiluminescent reaction of lipid peroxidation.2. Materials and methods