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Interaction of a synthetic peptide based on the neutrophil-derived antimicrobial protein CAP37 with dipalmitoyl-phosphatidylcholine membranes

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Abstract

CAP37, a cationic antimicrobial protein of M_r 37 kDa is constitutively expressed in human neutrophils. A synthetic peptide, CAP37 P_{20-44}, corresponding to amino acid residues 20 through 44 of the native CAP37 molecule has been shown to mimic the antimicrobial activity of the native protein. An analog of peptide CAP37 P_{20-44} was synthesized in which the cysteine residues at positions 26 and 42 were replaced with serine residues (CAP37 $P_{20-44Ser}$). This resulted in a peptide that no longer exhibited bactericidal activity. The effect of different concentrations of the active CAP37 peptide, CAP37 P_{20-44}, and its inactive analog, CAP37 $P_{20-44Ser}$, on artificial lipid membranes composed of dipalmitoyl phosphatidylcholine (DPPC) was studied using small-angle X-ray scattering and differential scanning calorimetry. The results indicated that CAP37 P_{20-44} perturbs the periodicity of the lamellar structure as shown by small angle X-ray diffraction, while the effect of the inactive peptide is not as strong. Differential scanning calorimetry further confirms that CAP37 P_{20-44} interacts with lipid membranes as indicated by increased width of the transition and decreased peak height. Moreover, it completely abolishes the pretransition temperature of the DPPC membranes. The effect of the inactive peptide, CAP37 $P_{20-44Ser}$ on the thermotropic properties of DPPC was small. These studies suggest that CAP37 perturbs the lamellar structure of lipid bilayers and further suggests that the antibiotic action of the molecule may be through its interactions with the lipid components of the Gram negative bacterial membrane. © 1997 Elsevier Science B.V.

Keywords: Lipid membrane; Small-angle X-ray scattering; CAP37; Differential scanning calorimetry

1. Introduction

CAP37 (Cationic antimicrobial protein of molecular weight of 37 kDa) is a multifunctional protein first isolated from the granules of human polymorphonuclear leukocytes or neutrophils (PMN) [1].

Abbreviations: DPPC: Dipalmitoyl-phosphatidylcholine; DSC: Differential scanning calorimetry; LPS: Lipopolysaccharide; PMN: Polymorphonuclear leukocyte; CAP37: Cationic antimicrobial protein of 37 kDa

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CAP37 was initially recognized for its potent bactericidal activity against Gram negative bacteria [2]. Recently, we have demonstrated that in addition to its antibiotic action, CAP37 is a strong chemoattractant for monocytes [3] and is capable of binding lipopolysaccharide (LPS) or endotoxin [4]. We have cloned and sequenced the gene for CAP37 and shown that it is a glycoprotein of 222 aminoacids [5,6]. It has strong homology with elastase (45%) and cathepsin G (32%), two serine esterases associated with PMN granules [3]. In an attempt to delineate the structure-function activity of the peptide, we synthesized overlapping peptides based on the amino acid sequence of the native CAP37 protein [4]. These peptides were tested for bactericidal activity against the Gram negative organism *Salmonella typhimurium*. One of these peptides based on amino acid residues 20 through 44 of native CAP37 (CAP37 P_{20-44}), demonstrated strong antibiotic activity [4], suggesting that this domain was probably the principal bactericidal domain of the molecule. Furthermore, our results suggested that CAP37 P_{20-44} was capable of binding LPS, indicating that both the antimicrobial and LPS binding domains of the protein were coincident [4]. We speculated that the binding of the cationic peptide to the LPS molecule initiated its antimicrobial activity. To determine the residues which were critical for these activities, we synthesized various analogs of peptide CAP37 P_{20-44}, which included replacements of certain amino acid residues. One such peptide consisted of replacing each of the cysteine residues at positions 26 and 42 by serines. The resulting peptide, CAP37 $P_{20-44Ser}$, was inactive in the bactericidal assay. Thus, it appeared that both cysteines which form a disulfide bond were critical for the maximal antibiotic activity of the protein [4].

This study was undertaken to evaluate the effect of the active CAP37 P_{20-44} peptide on model lipid membranes. Lipid bilayers serve as model membranes because their components and structure are similar to biological membranes. The interaction of CAP37 P_{20-44} peptide with DPPC lipid membranes was studied using differential scanning calorimetry (DSC) and small-angle X-ray diffraction. CAP37 P_{20-44} associated with and perturbed the periodicity of the membranes and affected the thermal transition of the bilayers. In comparison, CAP37 $P_{20-44Ser}$ which has markedly reduced antibiotic activity has a smaller effect than CAP37 P_{20-44} on the thermal and structural properties of DPPC as is shown by both DSC and X-ray diffraction.

2. Materials and methods

2.1. Lipid membranes

Dipalmitoyl–phosphatidyl choline, (DPPC > 99% pure) was purchased from Avanti, Birmingham, AL. Lipid membranes were prepared by dissolving 100 mg of DPPC in 50 ml chloroform. Varying concentrations of CAP37 P_{20-44} or CAP37 $P_{20-44Ser}$ (0–13% w/w) were dispersed in 10 ml of acetone and this mixture was added to the chloroform solution of DPPC and the solvent was removed through evaporation. Distilled water at a water/lipid ratio 3:1 was added to the dry lipid–peptide complex.

2.2. Peptide synthesis

Peptides were synthesized by solid-phase synthesis on an Applied Biosystems model 430A peptide synthesizer. Phenylacetamidomethyl copoly(styrene/divinylbenzene) resins and the tert-butoxycarbonyl (Boc)-protected amino acids (Bachem, PA) were used as previously described [4]. The Boc-amino acids in the peptides were double-coupled to achieve coupling efficiencies typically higher than 99.5%. Peptides were purified using reverse-phase high pressure liquid chromatography on an Aquapore RP-300 C_8 silica column (1×10 cm), 20 μm particle size and 300 Å pore size. The mobile phase consisted of acetonitrile and 0.1% of aqueous trifluroacetic acid. The purity and the integrity of the peptides were confirmed by amino acid sequencing and composition [4]. The mass of the peptides was confirmed by mass spectrometry, as previously reported [4].

Two peptides were used in this study. The first, was a peptide based on amino acid residues 20 through 44 of the native CAP37 molecule, designated CAP37 P_{20-44} and shown to have antibiotic and LPS-binding activity [4]. The second peptide CAP37 $P_{20-44Ser}$, was synthesized, such that serine residues replaced the cysteine residues at positions 26 and 42.

This peptide lacked antibiotic activity as previously demonstrated [4].

2.3. Differential scanning calorimetry and small angle X-ray diffraction

For DSC studies, the mixture consisting of DPPC with or without peptide was weighed in DSC crucibles and fully hydrated with a known quantity of water. Then, the crucibles were hermetically sealed. Each sample was scanned a minimum of three times until identical thermograms were obtained. Differential scanning calorimetry studies were performed on a Mettler 3000. The scanning rate was 2°C min^{-1} and the instrument was equilibrated using indium and fully hydrated DPPC.

The small angle X-ray experiments were conducted using the Oak Ridge National Laboratory, ORNL 10 m SAXS instrument [7], with Cu Kα radiation ($l = 1.54$ Å) and a 20×20 cm^2 position sensitive detector with each virtual cell element about 3 mm apart. The sample to detector distance was 1.495 m. The scattering intensity was stored in a 64×64 data array. Corrections were made for instrumental backgrounds, dark current due to cosmic radiation and electronic noises, and detector nonuniformity and efficiency (via an Fe^{55} radioactive standard which emits γ-rays isotropically) on a cell-by-cell basis. The data were azimuthally averaged in the Q range: 0.1 $nm^{-1} < 4.0$ nm^{-1}, $Q = 4\pi/\lambda)\sin(\theta/2)$ where λ is the X-ray wavelength and q is the scattering angle [8]. The hydrated lipid–peptide complex was placed in holders having windows sealed with Capton^{Tm} film.

3. Results and discussion

The DSC curve of pure DPPC was compared with the DSC curves of DPPC with 3, 5, and 12% w/w CAP37 P_{20-44} and CAP37 $P_{20-44Ser}$ in Figs. 1–3, respectively. A concentration of 3% CAP_{20-44} abolished the pretransition and significantly decreased the height and increased the width at the half-height ($\Delta T_{1/2}$) of the main transition (Fig. 1) reflecting a loss in cooperativity [9]. Increasing the amount of CAP37 P_{20-44} to 5% (Fig. 2) or 12% (Fig. 3) further

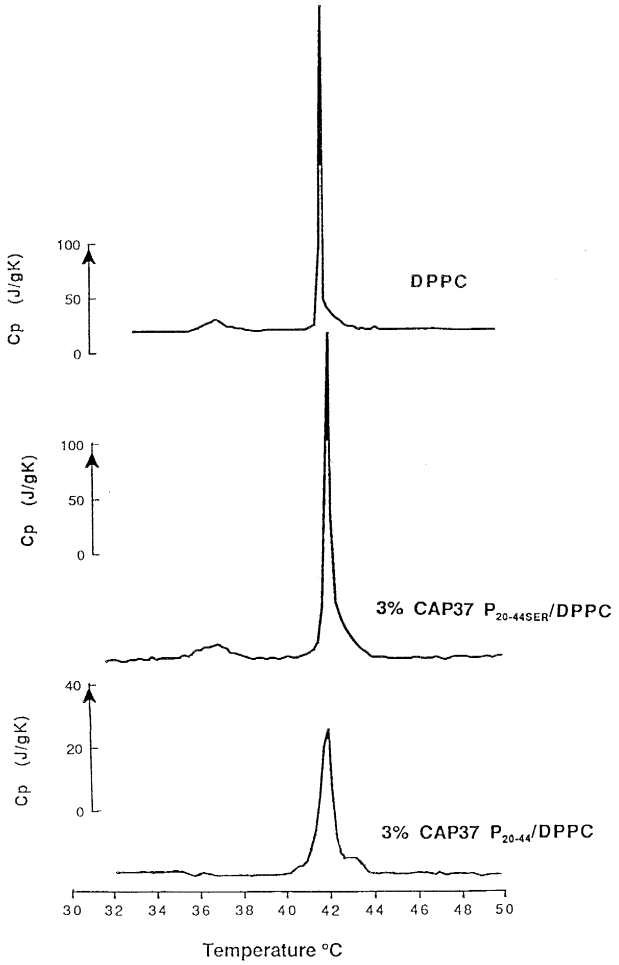


Fig. 1. DSC scans of pure DPPC, DPPC with 3% CAP37 $P_{20-44Ser}$ and DPPC with 3% CAP37 P_{20-44}. Note the difference in Cp scales.

reduced the height and increased the width of the transition, $\Delta T_{1/2}$. On the other hand, CAP37 $P_{20-44Ser}$ at a concentration of 3% (Fig. 1) or 5% (Fig. 2) preserved the pretransition, and the shape of the main transition remained similar to pure DPPC. However, the height of the main transition was reduced. Although 12% of the inactive CAP37 $P_{20-44Ser}$ (Fig. 3) further increased the width and reduced the height of the main transition peak of DPPC, the effect was not as dramatic as the effect of the active peptide, CAP37 P_{20-44}.

Differential scanning calorimetry studies the thermotropic aspect of protein–lipid interaction by examining the change in the melting point (T_m), the en-

thalpy and the shape of the DSC trace. During heating, the lipid initially undergoes a pretransition from an ordered 'gel' state, where the lipids are ordered and tilted (Lβ′ state) to the Pβ′ where the lipids are still ordered and in a gel state but the tilt is minimal. The pretransition occurs before the main transition and is small compared to the main one. During the main transition lipids undergo transition from the ordered state Pβ′ to a 'fluid' disordered state, Lα [10]. From the DSC data it is obvious that CAP37 P_{20-44} disturbs the main transition peak of DPPC, while CAP37 $P_{20-44Ser}$ which is the inactive peptide

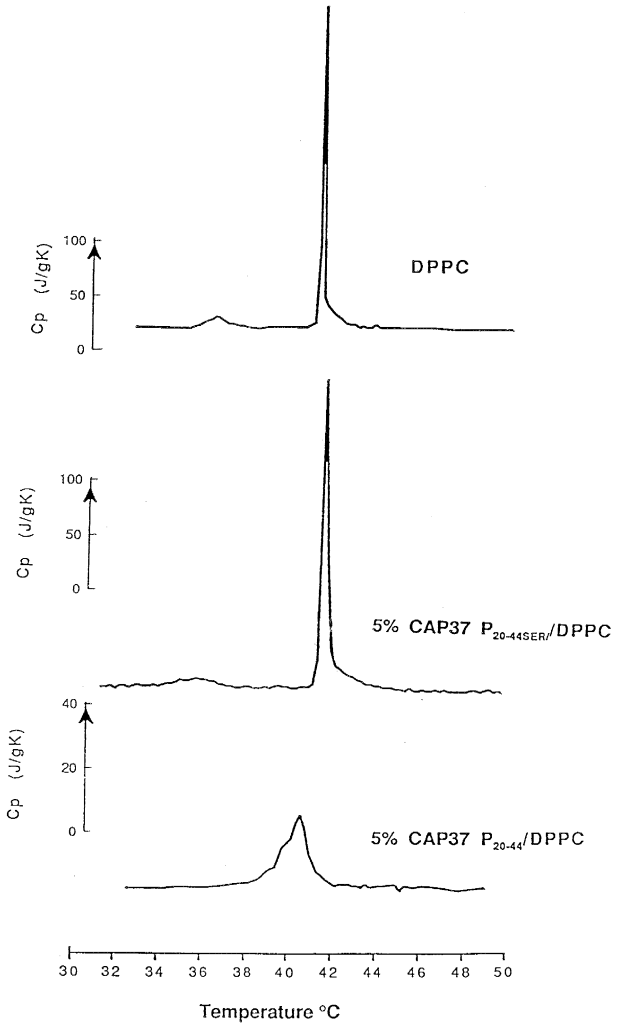


Fig. 2. DSC scans of pure DPPC, DPPC with 5% CAP37 $P_{20-44Ser}$ and DPPC with 5% CAP37 P_{20-44}. Note the difference in Cp scales.

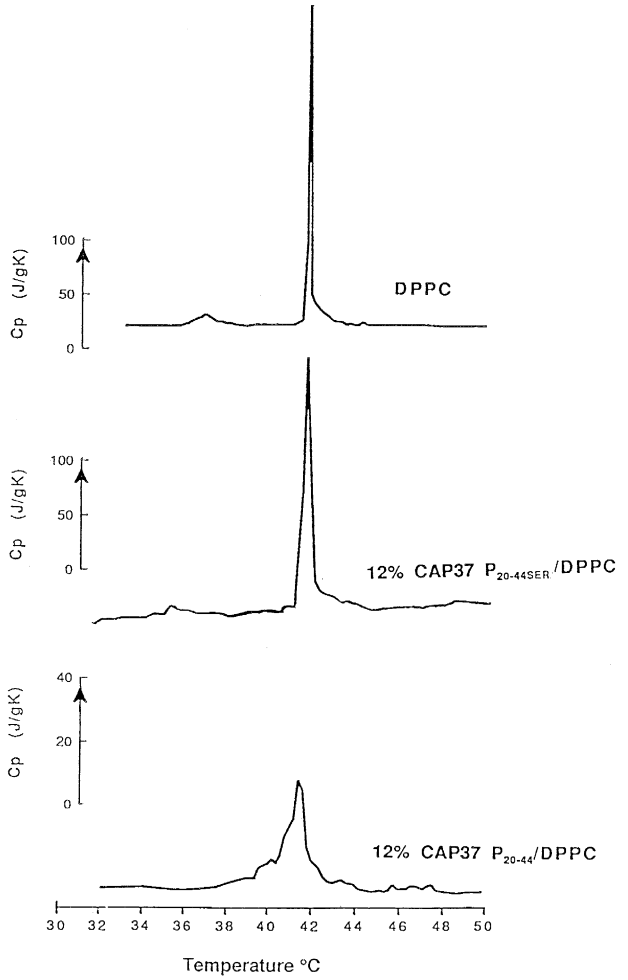
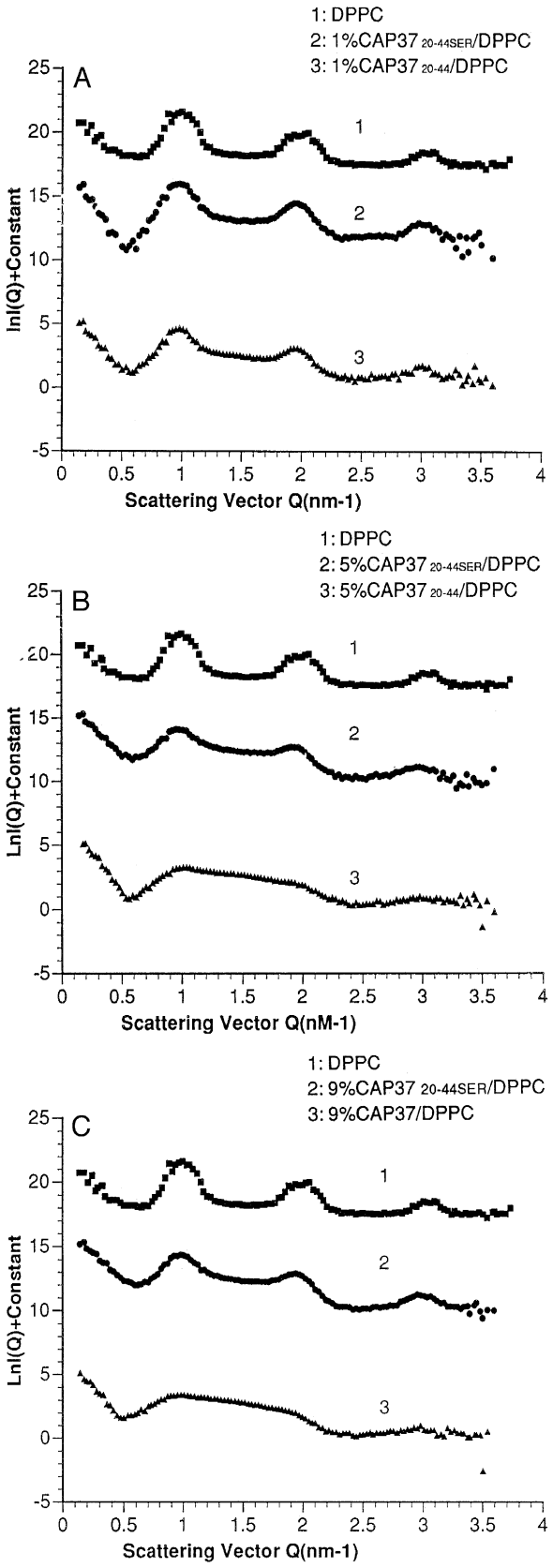


Fig. 3. DSC scans of pure DPPC, DPPC with 12% CAP37 $P_{20-44Ser}$ and DPPC with 12% CAP37 P_{20-44}. Note the difference in Cp scales.

does not significantly disturb the transition of the DPPC. In general, pretransition is very sensitive in the presence of impurities and it is abolished even at small quantities of impurities [9]. In the case of CAP37 $P_{20-44Ser}$, however, the pretransition is not abolished even at 5%, although there is a shift to a lower temperature. Neither CAP37 P_{20-44} nor CAP37 $P_{20-44Ser}$ affects the T_m of DPPC more than 1°C.

Small-angle X-ray diffraction spectra of pure DPPC and DPPC with different concentrations of CAP37 P_{20-44} and CAP37 $P_{20-44Ser}$ at 25°C are shown in Fig. 4. The pure DPPC pattern of X-ray spectrum reveals its lamellar structure, because the peaks are at equal



distances from each other (Fig. 4). Comparing the X-ray spectra of DPPC and DPPC with 1% CAP37 $P_{20-44Ser}$ or CAP37 P_{20-44} it can be noticed that the CAP37 P_{20-44} has the least sharp peaks (Fig. 4a). Furthermore, CAP37 P_{20-44} above 5% w/w flattens the DPPC peaks significantly and reduces the peak height so that the first and the second peak of DPPC have less intensity and their width is increased so that the peaks overlap. Thus CAP37 P_{20-44} interacts strongly with the lipid bilayer and causes irregularities which perturb it. Although CAP37 $P_{20-44Ser}$ increases the width of the DPPC peaks, the effect is not as strong as the CAP37 P_{20-44} effect. Small angle X-ray diffraction shows that CAP37 P_{20-44} perturbs, in a concentration related manner, the peaks of the DPPC. Thus, the X-ray findings support those obtained by DSC showing that CAP37 P_{20-44} interacts strongly with DPPC.

Overall, the native CAP37 molecule is basic, having 23 positively charged residues as opposed to 15 acidic residues [6]. The hydrophobicity plot of native CAP37 indicates a protein with alternating hydrophobic and hydrophilic domains, with one extended hydrophobic stretch between amino acids 26 and 58 [6]. CAP37 P_{20-44} which is comprised of amino acids NQGRHFCGGALIHARFVMTAASCFQ has a charge of +2 at neutral pH and is strongly hydrophobic [4], since 56% of its amino acid residues are hydrophobic. In addition, the active antimicrobial structure of the peptide requires the potential to form an intramolecular disulfide bond. Our preliminary data, thus suggest that at least for peptide 20–44, the antibiotic activity is probably due to a combination of charge and hydrophobicity, and that the presence of cysteines is very important [4]. A characteristic feature of antibiotic peptides such as the insect cecropins, is the requirement of an α-helical structure which helps them form voltage dependent ion channels in bacterial plasma membranes [11]. It has been

Fig. 4. Small angle X-ray diffraction spectra of DPPC (■), DPPC with CAP37 $P_{20-44Ser}$ (●) and DPPC with CAP37 P_{20-44} (▲). Pure DPPC, DPPC with 1% CAP37 $P_{20-44Ser}$ and DPPC with 1% CAP37 P_{20-44} (a); pure DPPC, DPPC with 5% CAP37 $P_{20-44Ser}$ and DPPC with 5% CAP37 P_{20-44} (b); pure DPPC, DPPC with 9% CAP37 $P_{20-44Ser}$ and DPPC with 9% CAP37 P_{20-44} (c).

demonstrated that for strong candidacidal activity, salivary histatins also form helices [12]. Magainins, antibacterial peptides obtained from frog skins, also form α-helices, and work through perturbation of membrane functions [13]. The mammalian defensins, unlike the above, tend to be composed of β-sheets, with no disposition to form α-helices [13,14]. A computer prediction (Chou–Fasman calculation) for CAP37 P_{20-44}, suggests that the α-helix model could be adjusted, such that beginning at Ala29 most of the hydrophobic residues would be distributed so as to closely conform to an ideal amphipathic helix. The glycine residues at the amino terminus of the peptide probably serve as helix breakers. Our current studies do not enable us to confirm that the mode of cytotoxicity is through formation of pores in the bacterial membrane. However, both the DSC and X-ray studies strongly indicate that CAP37 P_{20-44} perturbs the lamellar structure of lipid bilayers suggesting that the antibiotic action of CAP37 is through the interaction with the lipid components of the Gram negative bacterial membrane.

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