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Lipid composition is an important determinant of antimicrobial activity of alpha-melanocyte stimulating hormone

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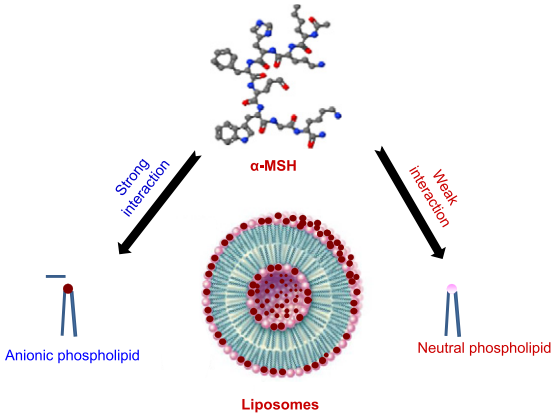
HIGHLIGHTS

- α-MSH preferentially interacts with anionic vesicles.
- Interaction with vesicles does not induce any secondary structure in α-MSH.
- α-MSH causes permeabilization and lysis of vesicles having negatively charged lipid.
- Both permeabilization and lysis by α-MSH are dependent on peptide concentration.

GRAPHICAL ABSTRACT



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ABSTRACT

We have reported strong antimicrobial activity of cationic neuropeptide α-MSH against *Staphylococcus aureus*. Clinical *S. aureus* isolates non-susceptible to the peptide had higher amount of cationic phospholipid. To elucidate the molecular basis of lipid selectivity and antimicrobial activity of α-MSH, studies were carried out on SUVs having different combinations of neutral DMPC and anionic lipids DMPG to mimic mammalian and bacterial membrane. The peptide interacted with the DMPG containing vesicles only, as evident from the changes in Trp fluorescence. CD spectroscopy revealed that despite interaction, the peptide retained its native random coil structure. The perturbation of the vesicles caused by peptide interaction is strongly dependent on peptide concentration as seen both by DLS and Tb^{3+}/DPA based fluorescence leakage assay. Our data clearly demonstrate the preference of α-MSH to interact with anionic DMPG containing vesicles leading to significant permeabilization which is the molecular basis behind the selectivity of α-MSH for bacterial systems.

1. Introduction

Antimicrobial peptides (AMPs) have been hailed as a potential solution to the dearth of novel antibiotic development in the past 50 years. AMPs are produced by almost all organisms and are relatively small (6–100 amino acids) molecules of variable length, sequence and

Abbreviations: α-MSH, alpha-melanocyte stimulating hormone; DMPC, 1,2-dimyristoyl-sn-glycero 3 phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-glycerol; DLS, dynamic light scattering; CD, circular dichroism; SUV, small unilamellar vesicles; Tb^{3+}/DPA, Terbium/dipicolinic acid.

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structure with activity against a wide range of microorganisms. Despite these variations, the key features that render them to exhibit microbicidal activity are i) their cationicity and ii) their binding and adoption of secondary structural conformation in membrane environments [1]. These characteristics allow them to attach to and insert into the bacterial membrane rather than host membranes. Mostly all AMPs are understood to target the bacterial plasma membrane directly rather than through specific protein receptors [2]. Therefore the phospholipid composition, in particular the net charge of the membranes plays a vital role in determining the antimicrobial activity of the AMPs [3]. A number of studies have recently demonstrated that the introduction of positive charges on bacterial surface lowers the electrostatic interaction between AMPs and bacteria, thus increasing bacterial resistance [3]. Several liposomal models have been proposed to explain the bacterial membrane damage by AMPs and it has been shown that the threshold concentration of AMPs is an essential factor to achieve their antimicrobial activity. Below a threshold concentration, there is little activity despite binding; but when the concentration exceeds the threshold, the activity is at its maximum [4]. For example, in a very recent model membrane study it was shown that at lower peptide-lipid molar ratio of 1:250–1:350, the activity of human α-defensin HNP-1 is prevalently localized on membrane surface, which can bring to a thinning and destabilization of phospholipid bilayer. On increasing peptide concentration to 1:20, penetration and pore formation is the more suitable mechanism of action [5]. Despite numerous experimental and simulation studies on the mechanism of action of various AMPs, the underlying mechanism, like how AMPs perturb the membrane, remains enigmatic [6]. There are studies where certain membrane-active peptides like cecropins, magainins and mellitins have been demonstrated to permeabilize model membrane systems, leading to leakage of fluorescent dyes from unilamellar vesicles, or induce transport across lipid bilayers [2].

Alpha-melanocyte stimulating hormone (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), is an endogenous linear tridecapeptide known for its role in regulating skin pigmentation in vertebrates [7]. Beside this primary role, it also has many other biological functions like anti-inflammatory, neuroprotective, antipyretic and anti-apoptotic effects through binding to melanocortin G protein-coupled receptors (MCR) [8–11]. Recently we have demonstrated the antimicrobial activity of this cationic neuropeptide against the opportunistic pathogen *Staphylococcus aureus* (*S. aureus*) in vitro and in vivo as well. Furthermore we demonstrated that bacterial membrane damage is the primary mechanism of antibacterial activity of the peptide [12,13]. Very recently, our group also showed that α-MSH was least toxic to the mammalian cell line and it did not cause hemolysis of RBCs [14]. In another recent study by our group, it was found that clinical isolates of *S. aureus* that were non-susceptible to α-MSH had comparatively more amount of cationic phospholipid, lysylphosphatidylglycerol (LPG) and less amount of anionic phospholipid, phosphatidylglycerol (PG) in their membrane [15]. Taken together it looks like α-MSH preferentially interacts with negatively charged membrane and like any other cationic AMP, charge seems to be an important factor in the antimicrobial action of α-MSH.

It is postulated that permeabilization of the membrane by AMPs depends on peptide structure and phospholipid matrix. A number of studies have been reported on the behavior of α-MSH and its analogues in model membranes made of differentially charged phospholipids [16–19]. Most of them elucidated the structure-activity of the melanotropins and indicated that the structure and stability of α-MSH in negatively charged membrane were substantially different from those of the peptide in solution; being stabilized in a specific conformation that could be important to elicit its biological activity [18,20–22]. Previous reports have clearly indicated that α-MSH was able to interact and penetrate membranes. A two-step process was described: an initial electrostatic interaction between the positively charged peptide with negatively charged lipids of the membrane by which it reaches an appropriate local concentration and then the penetration of the peptide in the hydrophobic core of the bilayer [20,21,23,24]. The secondary structure of α-MSH in membrane-mimetic solvent 2,2,2-trifluoroethanol (TFE) is reported to be random coil [25]. However, the activity of α-MSH in different combinations of anionic and neutral lipids has not been explored. Taking these observations into consideration, in the present study we focused to understand the role of lipid composition in antibacterial activity of α-MSH. Here, we have studied in detail the interaction of α-MSH in simple membrane mimetic like Small Unilamellar Vesicles (SUVs) that are easy to prepare with reproducible average diameter and diameter size distribution. SUV's were formed by the phospholipid, dimyristoylphosphatidylcholine (DMPC) with zwitterionic headgroups i.e. with no net charge and dimyristoylphosphatidylglycerol (DMPG) with anionic headgroups. DMPC was chosen to mimic surface membrane of mammalian cells because the lipid is stable to oxidation and readily hydrates in water forming lamellar phases at physiological pH and temperatures. DMPG was chosen since phosphatidylglycerol is absent in eukaryotic plasma membranes but is ubiquitous and abundant in bacterial membranes. We have prepared SUVs composed of different ratios of DMPC and DMPG. This was done to modulate the negative charge on the vesicle surface. Carefully designed controls using dynamic light scattering (DLS) were used to look at the stability of the vesicles under the experimental conditions studied. Stability was assessed by the invariance of the average diameter and the size distribution with increasing standing time of the sample. DLS was also used to observe changes in the average vesicular diameter and size distribution, if any, in the presence of varying concentration of the peptide. We have measured the changes in the tryptophan (Trp) fluorescence of α-MSH in the various combinations of DMPC and DMPG SUVs to see if there is any difference in the interaction of the peptide with these vesicles. Circular dichroism (CD) spectroscopy was used in the far-ultraviolet region to monitor the changes in the secondary structures of the peptide in different membrane environments. Finally, we have performed the leakage assay with terbium/dipicolinic acid (Tb^{3+}/DPA) in order to examine the effect of increasing concentrations of α-MSH on the rate of permeabilization of differentially charged phospholipids. This assay monitors the rate and extent of the leakage of the inner aqueous content of the vesicles into the bulk aqueous phase, which in turn measures the perturbing effect of the peptide on vesicles. The detail of the leakage assay is provided in the next section.

2. Materials and methods

DMPC [1,2-dimyristoyl-*sn*-glycero-3-phosphocholine] and DMPG [1,2-dimyristoyl-*sn*-glycero-3-phosphor-*rac*-(1-glycerol) sodium salt], TritonX-100 (ultrapure), dipicolinic acid (DPA), terbium (Tb^{3+}) chloride, sephadex G-50, and alpha-melanocyte stimulating hormone (α-MSH) were purchased from Sigma-Aldrich (St. Louis, MO). The concentration of α-MSH was determined spectrophotometrically (Shimadzu UV-2450 spectrophotometer). 2-[tris(hydroxymethyl)-methylamine]-1-ethanesulfonic acid (TES buffer) and sodium ethylene diamine tetra-acetate (EDTA sodium salt) were purchased from SRL (India), and all were used without further purification.

2.1. Preparation of SUVs

Small unilamellar vesicles of DMPC, DMPG and combinations of the lipids up to the ratio of 50:50 DMPC:DMPG were prepared by the method of sonication as described elsewhere [26]. Briefly, phospholipids were weighed and dissolved in 2:1 (v/v) chloroform:methanol solution and the solvent was evaporated by purging argon to prepare the lipid film. It was then dried overnight in a vacuum desiccator at −20 °C. The dried film was hydrated and swelled in 10 mM TES, pH 7.4 for CD spectra and fluorescence measurements. For DLS study, SUVs were prepared by dissolving lipid films in double filtered 10 mM MOPS (3-N-morpholino propanesulfonic acid) buffer. For leakage assay, dried film was hydrated and swelled in 10 mM TES and 60 mM NaCl at pH 7.4 for $TbCl_3$ (8 mM)