Biochemical and Biophysical Research Communications 397 (2010) 1–4

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Topology and dynamics of melittin within the liposome revealed by a combination of mass spectrometry and chemical modification

Kazumi Saikusa, Yo Kono, Shunsuke Izumi *

Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan

ARTICLE INFO

Article history:
Received 30 March 2010
Available online 14 April 2010

Keywords:
Melittin
Terminal topology
Dynamics
MALDI-TOF MS
MALDI-QIT-TOF MS/MS
Acetylation

ABSTRACT

The topology and dynamics of melittin within the liposome were investigated by a mass spectrometry coupled with acetylation. The MALDI-TOF MS and MALDI-QIT-TOF MS/MS analyses revealed that only N-terminal amine of melittin was dominantly acetylated in the presence of liposome although all of four primary amines were completely and rapidly acetylated in aqueous solution. This result indicates that melittin adopts the N-terminal-outside transmembrane topology within the liposome. The time course of acetylation followed the first-order kinetics at any examined temperatures (6–30 °C). The rate constant was less than that of the acetylation of melittin in aqueous solution. The activation energy for acetylation (74 kJ mol^{-1}) was comparable to that for dissociation of a lipid monomer from the membrane, suggesting a float-like longitudinal motion of melittin within the liposome. These results demonstrate that a mass spectrometry combined with chemical modification is very efficient way for clarifying the topology and dynamics of peptides bound to the membrane.

1. Introduction

Melittin is a hemolytic peptide consisting of 26 amino acid residues isolated from the honeybee *Apis mellifera* [1]. The N-terminal segment of 20 residues is highly hydrophobic, whereas the C-terminal segment is highly cationic because it contains two lysine and two arginine residues. This amphiphilic property of melittin allows it to associate spontaneously with natural and artificial membranes [2,3] as commonly observed in many membrane-binding peptides and transmembrane proteins. Therefore, melittin has been used as a convenient model peptide for investigating the protein–membrane interactions.

Melittin exists in a monomer of essentially random conformation in aqueous solutions [2–5]. However, at high concentrations of peptide and salt or at high pHs, melittin adopts an α-helical conformation and aggregates into a tetramer [2–8]. Melittin also adopts an α-helical conformation when bound to micelles or liposomes of varying lipid composition [2,3,9–15]. The topology of the helical domains within the membrane is an important issue to be clarified because it is closely related to the dynamics (such as lateral diffusion and reorientation) and function of melittin. Solid-state NMR study revealed that the kink angle between the N- and C-terminal helical rods of melittin was 140° or 160° in the membrane of dimyristoylphosphatidylcholine and that melittin laterally diffused tilting the N- and C-terminal helical axes to the membrane by 30° and 10°, respectively [15]. However, according to the oriented circular dichroism analysis, the axes of helical rods changed depending on the peptide concentration in the lipid bilayers (peptide–lipid molar ratio). Namely, the orientation of the helices is parallel and perpendicular to the plane of membrane at low and high peptide concentrations, respectively [14,16]. On the other hand, fluorescent-labeling or spin-labeling techniques suggested that the helix axes of melittin adopt a pseudoparallel orientation with a tilt angle of about 70° to the membrane surface and the N-terminal is flexible, whereas the motions of Lys7 and C-terminal are restricted in the membrane [17,18]. These results provide useful information for understanding the molecular mechanism of melittin–membrane interactions. However, the topology and dynamics of melittin within the membrane remains controversial probably due to the analytical limitation of each method.

A novel method to address the topology and dynamics of melittin is a mass spectrometry coupled with chemical modification. Since four primary amines (N-terminal, Lys7, Lys21, and Lys23) of melittin are easily acetylated, a precise mass spectrometry allows us to detect the extent and site of acetylation on melittin molecule as a function of acetylation time at various temperatures, which should give new information on the topology and dynamics of melittin within the membrane. This chemical modification method is similar in principle to the hydrogen/deuterium (H/D) exchange analysis of protein dynamics. Our group has confirmed that a mass spectrometry coupled with H/D exchange is a powerful tool for monitoring the site-specific fluctuation of dihydrofolate reductase and 70S ribosomal proteins [19,20]. However, since

* Corresponding author. Fax: +81 82 424 7327.
E-mail address: sizumi@sci.hiroshima-u.ac.jp (S. Izumi).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.04.020

deuterium oxide passes through the membranes, it was expected that the H/D exchange analysis would be difficult for the dynamics of melittin within the liposome.

In the present study, we investigated the terminal topology and dynamics of melittin within the liposome using a mass spectrometry combined with acetylation. The N-terminal amine and the lysine residues of melittin were acetylated with *N*-hydroxysulfosuccinimidyl acetate, which is a water-soluble and membrane-impermeable reagent, in the presence or absence of liposome [21,22]. It was found that some amine residues buried in the hydrophobic core of the membrane cannot be acetylated and melittin adopts the N-terminal-outside topology. Further, the activation energy for acetylation suggested a float-like longitudinal motion of melittin within the liposome. To our knowledge, this is the first application of mass spectrometry combined with chemical modification to the study of the topology and dynamics of melittin within the membrane.

2. Materials and methods

2.1. Materials

Melittin from the honeybee venom (*A. mellifera*) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) and used without further purification. Egg-yolk phosphatidylcholine, cholic acid, α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid were products of Sigma–Aldrich Co. 1,2-Diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) and *N*-hydroxysulfosuccinimidyl acetate (NHSSAc) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and Pierce/Thermo-Fisher (Rockford, IL, USA), respectively.

2.2. Liposome preparation

Liposomes of phosphatidylcholine were prepared by the cholate-dialysis method [23]. Phosphatidylcholine dissolved in chloroform was dried under a stream of N_2 gas and evacuated overnight to remove the residual solvent. Three hundred microliters of 10% sodium cholate was added to the dried phosphatidylcholine at a molar ratio of phospholipid to cholate of 1:3. The phospholipid–cholate mixture was ultrasonicated at 4 °C for 3 min with a sonicator equipped with a cup-horn probe (SONIFIER 450, Branson, Connecticut, CT, USA) and stirred with a vortex mixer for 2 min under an Ar atmosphere until the suspension became clear. A phosphate buffer containing glycerol was added to the phospholipid–cholate mixture to a final volume of 1.0 ml at a final concentration of 500 mM potassium phosphate (pH 7.2) and 20% glycerol. The obtained phospholipid–cholate solution was dialyzed for 3 h against 500 mM potassium phosphate (300 ml) containing 20% glycerol and 0.3% sodium cholate and subsequently overnight against 50 mM potassium phosphate (pH 7.2) containing 20% glycerol.

2.3. Acetylation of melittin within the liposome or micelle

Melittin was mixed with the liposome solution at a molar ratio of phosphatidylcholine to melittin of 1500:1 and the mixture was incubated for 2 h on ice before acetylation. For comparison, the melittin–micelle mixtures were prepared by dissolving DHPC and melittin (molar ratio of 1500:1) in 50 mM potassium phosphate buffer (pH 7.2) at a final DHPC concentration of about 13 mM, which was higher than its critical micelle concentration. The acetylation reagent, NHSSAc, was dissolved in distilled water at a concentration of 1 mg/ml. The acetylation of melittin was initiated by mixing 2.82 μl of fleshly prepared NHSSAc solution with 12 μl of melittin–liposome or melittin–micelle mixtures at a molar ratio of melittin to NHSSAc of 1:50. After various intervals of incubation (2–180 min) at a given temperature (6–30 °C), the reaction was terminated by adding 6.6 μl of 5 mM NH_4HCO_3. A portion (1.0 μl) of the reaction mixture was mixed with a matrix solution (0.5 μl) to measure the mass of acetylated melittin.

2.4. Mass spectrometry

The extent and site of acetylation of melittin were analyzed with MALDI-TOF MS (AXIMA-CFR, Shimadzu, Kyoto, Japan) and MALDI-QIT-TOF MS (AXIMA-QIT, Shimadzu, Kyoto, Japan). α-Cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were used as the matrix in MALDI-TOF MS and MALDI-QIT-TOF MS, respectively, at a concentration of 10 mg/ml in 50% acetonitrile involving 0.1% trifluoroacetic acid. Above mentioned drop of the sample–matrix solution was added to the matrix (0.5 μl) on the target plate, air-dried, and then the target plate was introduced into the mass spectrometers. The sample was ionized with a pulsed N_2 laser at 337 nm and its mass was measured in reflector (positive ion) mode at 1×10^{-4} Pa. The number of shots per spectrum was 1000 in the mass range of *m/z* 2500–3500 for MALDI-TOF MS and 10,000 in the mass range of *m/z* 550–3500 for MALDI-QIT-TOF MS. The mass spectra were calibrated with an accuracy of ±60 ppm using angiotensin II ($[M+H]^+ = m/z$ 1046.54) and fragment 18–39 of adrenocorticotropic hormone ($[M+H]^+ = m/z$ 2465.20). The time course of acetylation was examined over the reaction time of 180 min at various temperatures of 6–30 °C and analyzed with the first-order reaction kinetics according to the H/D exchange method [19,20]:

$$D_t = D_\infty \times \exp(-kt) \quad (1)$$

where D_t and D_∞ are the fractions of non-acetylated amine residues at reaction time t and infinity, respectively; and k is the apparent first-order rate constant of acetylation.

3. Results and discussion

3.1. Terminal topology of melittin within the membrane

Melittin has four primary amines (N-terminal, Lys7, Lys21, and Lys23) that are susceptible to acetylation. Fig. 1 shows the MALDI-TOF mass spectra of melittin acetylated in the presence or absence

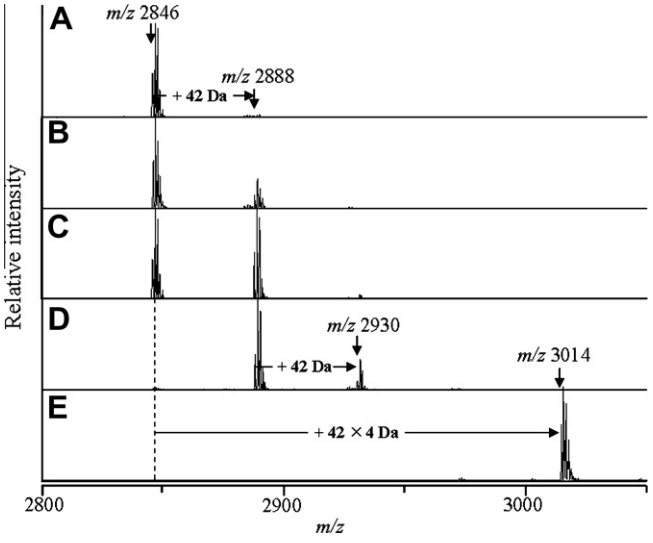

Fig. 1. MALDI-TOF mass spectra of melittins acetylated within the liposome (a–d) and in aqueous solution (e). Reaction time: (a) 2 min; (b) 13 min; (c) 30 min; (d) 180 min; (e) 5 min.

of liposome at room temperature. In the spectra of melittin acetylated within the liposome (Fig. 1a–d), three peaks were detectable at m/z 2846, 2888, and 2930 during the acetylation time of 180 min. The peak at m/z 2846 was due to protonated melittin molecule, and the peaks at m/z 2888 and 2930 were assigned to mono- and di-acetylated melittins, respectively, because these peaks shifted by 42 and 84 (42×2) Da compared to the protonated melittin molecule of m/z 2846. However, no such intermediately acetylated peaks were observed while the peak for tetra-acetylated melittin (m/z 3014) appeared even at 5-min acetylation in aqueous solution (Fig. 1e). Thus, any primary amines of melittin were completely and rapidly acetylated in aqueous solution, but melittin within the liposome was highly protected from acetylation.

In order to identify the acetylated residue of melittin, the peak at m/z 2888 was analyzed by MALDI-QIT-TOF MS/MS. Fig. 2 shows the MALDI-QIT-TOF MS/MS spectra for the peaks of m/z 2846 and 2888. The sequential fragment b ions (b_{21}, b_{22}, b_{23}, and b_{24}) and y ions (y_{15}, y_{16}, and y_{17}) were observed for both protonated and mono-acetylated melittins within the liposome. These b ions were ascribed to mono-acetylated fragment because they shifted by 42 Da relative to those of non-acetylated melittin. On the other hand, the y ions in mono-acetylated melittin did not show any shift from those of non-acetylated melittin. These results suggest that the acetylated residue is either N-terminal amine or Lys7 residue. To determine which of the two residues was acetylated, acetylated melittin was digested with trypsin. The MALDI-TOF mass spectrum of the tryptic fragment showed a peak at m/z 699, which corresponded to the acetylated fragment of residues 1–7. Since the C-terminal residue of this tryptic fragment is Lys7, Lys7 was excluded from the candidacy for acetylation site. The peak at m/z 699 was analyzed by MALDI-QIT-TOF MS/MS. The sequential fragment b ions (b_3, b_4, and b_5) were observed. These b ions were ascribed to mono-acetylated fragment because they shifted by 42 Da relative to those of the non-acetylated fragment of residues 1–7. Therefore, the acetylated residue can be N-terminal amine.

Next, to elucidate the orientation of melittin within the liposome, melittin was acetylated in the presence of micelle instead of liposome for 30 min at room temperature. The N- and C-terminal ionic residues of melittin would hardly penetrate into the loosely packed core of hydrocarbon chains in a spherical micelle [24]. The MALDI-TOF mass spectrum of melittin acetylated in the presence of micelle showed two peaks at m/z 2972 and 3014 (Supplementary Fig. S1b). These peaks were assigned to tri- and tetra-acetylated melittins, respectively. No peak for non- or mono-acetylated melittin was observed in contrast to the acetylation in the presence of liposome, although the rate of acetylation was very slow in the presence of micelle in comparison with in water (Supplementary Fig. S1). Therefore, these results indicate that melittin is bound around the surface of micelle, whereas it adopts a transmembrane orientation within the liposome. Our results approve of the study on the structure of melittin bound to micelle carried out by mass spectrometry combined with H/D exchange [13]. Consequently, we concluded that melittin adopts a transmembrane and N-terminal-outside topology within the liposome or membrane (Fig. 3).

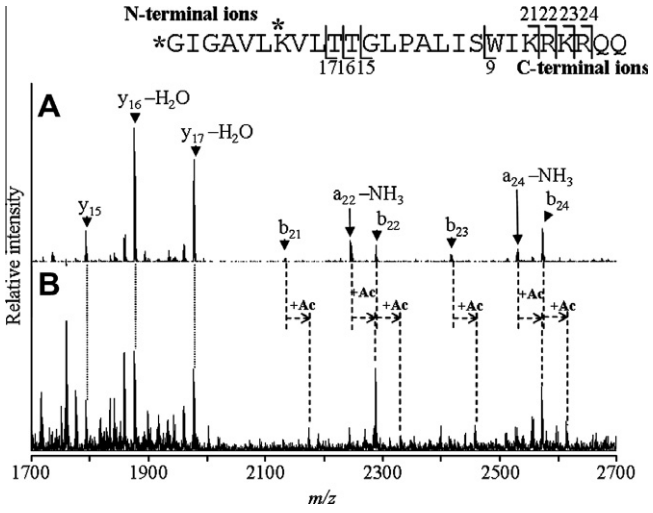

Fig. 2. MALDI-QIT-TOF MS/MS spectra of (a) protonated and (b) mono-acetylated melittins within the liposome.

3.2. Kinetics of acetylation of melittin within the liposome

As shown in Fig. 1a–d, the peak intensity of non-acetylated melittin (m/z 2846) decreases with an increase in acetylation time accompanying the increase in peak intensities of acetylated melittins. Kinetics of acetylation was investigated in more detail at various temperatures between 6 and 30 °C. Fig. 4a shows plots of the fraction of non-acetylated melittin against the acetylation time in the presence of liposome. In the ranges of acetylation time and temperatures, mono-acetylated melittin was dominantly observed and the fraction of more acetylated one was negligibly small. Evidently, acetylation of melittin followed the first-order kinetics with being enhanced at higher temperatures and most melittins remained without being acetylated within 2 min at any temperatures. As shown in Fig. 4b, the Arrhenius plot for acetylation showed a clear linear line with a slope corresponding to the activation energy of 74 kJ mol^{-1}. Yano and Matsuzaki reported the similar activation enthalpy (74 kJ mol^{-1}) for dissociation of a transmembrane model peptide, fluorophore-labeled 7-nitro-2-1,3-benzoxadiazol-4-y(NBD)-$(LALAAAA)_3$-NH_2, from the membrane [25]. The activation enthalpy for dissociation of a constituent lipid monomer from the phospholipid membrane was also estimated to be 75 kJ mol^{-1} from the transfer rate of NBD-phosphatidylcholine between two dioleoylphosphatidylcholine liposomes [26]. Since the activation energy is almost the same value as the activation enthalpy, the comparable activation energies which are observed for acetylation of melittin and dissociation of lipid or hydrophobic peptide strongly suggest that the acetylation is closely coupled with the exposure of melittin from the liposome.

Since the exposure of amine residues to the solvent is indispensable for acetylation and the exposed amines are rapidly acetylated, the acetylation kinetics of melittin within the liposome could be explained by the following model, which is similar to the H/D exchange reaction of protein in the closed and open forms:

$$M_b \underset{k_b}{\overset{k_e}{\rightleftarrows}} M_e \xrightarrow{k_a} M_a \qquad (2)$$

where 'M' represents melittin; the subscripts 'b' and 'e', and 'a' represent the 'buried', 'exposed', and 'acetylated' states of melittin, respectively; k_b, k_e, and k_a are the rate constants for each process in above equation. When $k_a > k_b$, the observed rate constant, k, is determined

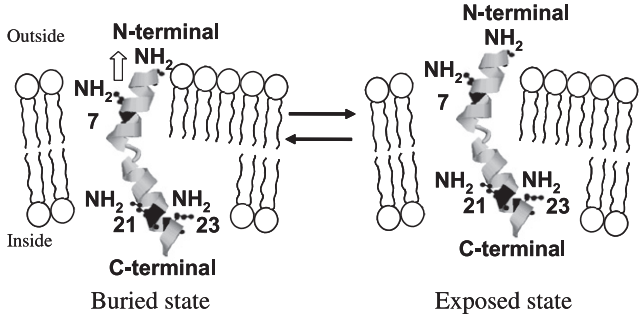

Fig. 3. Proposed model for the topology and dynamics of melittin within the liposome.

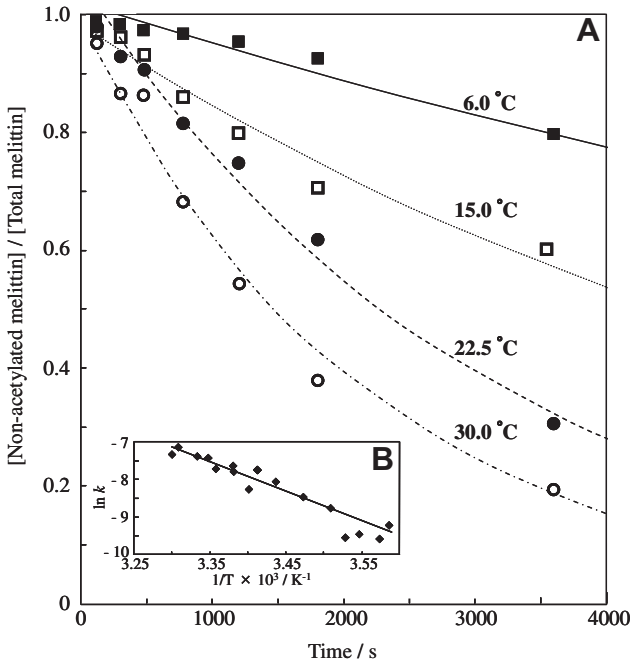

Fig. 4. (a) Time course of acetylation of melittin within the liposome at various temperatures. The fraction of non-acetylated melittin was calculated as the relative peak intensities of non-acetylated melittin ion to total melittin ions in MALDI-TOF mass spectra. (b) Arrhenius plot for the first-order rate constant of acetylation. The least-squares linear regression represents a slope corresponding to an activation energy of 74 kJ mol^{-1}.

by the rate of exposing melittin from the liposome, k_e. If $k_a < k_b$, the observed rate of acetylation is $(k_e/k_b)k_a$, and k is then proportional to the equilibrium constant between the buried and exposed states of melittin. Since the observed rate of acetylation of melittin was very slow within the liposome in comparison with the one in water (within the range of second) [27], the exposure of melittin from the liposome would be a rate-limiting process in its acetylation. It is highly possible that melittin is in a float-like longitudinal motion keeping the N-terminal-outside topology within the liposome.

As demonstrated in the present study, a mass spectrometry combined with chemical modification is very useful for elucidating the terminal topology and dynamics of melittin bound to liposome although more detailed assignments of acetylated residues are necessary. This method can be generally applicable to investigation of the topology and dynamics of membrane-binding peptides and transmembrane helices in intact cells because the recent development of mass spectrometry has realized a comprehensive analysis of constituent proteins in a cell [28,29].

Acknowledgments

We wish to thank the Natural Science Center for Basic Research and Development, Hiroshima University, for the use of AXIMA-CFR. We also thank the reviewer for his/her insightful and constructive comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.04.020.

References

[1] E. Habermann, Bee and wasp venoms, Science 177 (1972) 314–322.
[2] C.E. Dempsey, The actions of melittin on membranes, Biochim. Biophys. Acta 1031 (1990) 143–161.
[3] H. Raghuraman, A. Chattopadhyay, Melittin: a membrane-active peptide with diverse functions, Biosci. Rep. 27 (2007) 189–223.
[4] J.C. Talbot, J. Dufourcq, J.D. Bony, J.F. Faucon, C. Lussan, Conformational change and self association of monomeric melittin, FEBS Lett. 102 (1979) 191–193.
[5] J. Lauterwein, L.R. Brown, K. Wuthrich, High-resolution ^{1}H NMR studies of monomeric melittin in aqueous solution, Biochim. Biophys. Acta 622 (1980) 219–230.
[6] C.R. Dawson, A.F. Drake, J. Helliwell, R.C. Hider, The interaction of bee melittin with lipid bilayer membranes, Biochim. Biophys. Acta 510 (1978) 75–86.
[7] J. Bello, H.R. Bello, E. Granados, Conformation and aggregation of melittin: dependence on pH and concentration, Biochemistry 21 (1982) 461–465.
[8] A.F. Drake, R.C. Hider, The structure of melittin in lipid bilayer membranes, Biochim. Biophys. Acta 555 (1979) 371–373.
[9] S. Sui, H. Wu, Y. Guo, K. Chen, Conformational changes of melittin upon insertion into phospholipid monolayer and vesicle, J. Biochem. 116 (1994) 482–487.
[10] A.K. Ghosh, R. Rukmini, A. Chattopadhyay, Modulation of tryptophan environment in membrane-bound melittin by negatively charged phospholipids: implications in membrane organization and function, Biochemistry 36 (1997) 14291–14305.
[11] F. Inagaki, I. Shimada, K. Kawaguchi, M. Hirano, I. Terasawa, T. Ikura, N. Go, Structure of melittin bound to perdeuterated dodecylphosphocholine micelles as studied by two-dimensional NMR and distance geometry calculations, Biochemistry 28 (1989) 5985–5991.
[12] H. Vogel, Comparison of the conformation and orientation of alamethicin and melittin in lipid membranes, Biochemistry 26 (1987) 4562–4572.
[13] S. Akashi, K. Takio, Structure of melittin bound to phospholipid micelles studied using hydrogen–deuterium exchange and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, J. Am. Soc. Mass Spectrom. 12 (2001) 1247–1253.
[14] L. Yang, T.A. Harroun, T.M. Weiss, L. Ding, H.W. Huang, Barrel-stave model or toroidal model? A case study on melittin pores, Biophys. J. 81 (2001) 1475–1485.
[15] A. Naito, T. Nagao, K. Norisada, T. Mizuno, S. Tuzi, H. Saito, Conformation and dynamics of melittin bound to magnetically oriented lipid bilayers by solid-state ^{31}P and ^{13}C NMR spectroscopy, Biophys. J. 78 (2000) 2405–2417.
[16] F.Y. Chen, M.T. Lee, H.W. Huang, Evidence for membrane thinning effect as the mechanism for peptide-induced pore formation, Biophys. J. 84 (2003) 3751–3758.
[17] H. Raghuraman, A. Chattopadhyay, Orientation and dynamics of melittin in membranes of varying composition utilizing NBD fluorescence, Biophys. J. 92 (2007) 1271–1283.
[18] S. Haldar, H. Raghuraman, A. Chattopadhyay, Monitoring orientation and dynamics of membrane-bound melittin utilizing dansyl fluorescence, J. Phys. Chem. B 112 (2008) 14075–14082.
[19] T. Yamamoto, S. Izumi, K. Gekko, Mass spectrometry on segment-specific hydrogen exchange of dihydrofolate reductase, J. Biochem. 135 (2004) 17–24.
[20] T. Yamamoto, S. Izumi, K. Gekko, Mass spectrometry of hydrogen/deuterium exchange in 70S ribosomal proteins from *E. coli*, FEBS Lett. 580 (2006) 3638–3642.
[21] P.N. Lewis, J.G. Guillemette, S. Chan, Histone accessibility determined by lysine-specific acetylation in chicken erythrocyte nuclei, Eur. J. Biochem. 172 (1988) 135–145.
[22] X. Guo, P. Bandyopadhyay, B. Schilling, M.M. Young, N. Fujii, T. Aynechi, R.K. Guy, I.D. Kuntz, B.W. Gibson, Partial acetylation of lysine residues improves intraprotein cross-linking, Anal. Chem. 80 (2008) 951–960.
[23] S. Izumi, H. Kaneko, T. Yamazaki, T. Hirata, S. Kominami, Membrane topology of guinea pig cytochrome P450 17α revealed by a combination of chemical modifications and mass spectrometry, Biochemistry 42 (2003) 14663–14669.
[24] H. Hauser, Short-chain phospholipids as detergents, Biochim. Biophys. Acta 1508 (2000) 164–181.
[25] Y. Yano, K. Matsuzaki, Membrane insertion and dissociation processes of a model transmembrane helix, Biochemistry 41 (2002) 12407–12413.
[26] J.W. Nichols, Thermodynamics and kinetics of phospholipid monomer–vesicle interaction, Biochemistry 24 (1985) 6390–6398.
[27] G.W. Cline, S.B. Hanna, Kinetics and mechanisms of the aminolysis of *N*-hydroxysuccinimide esters in aqueous buffers, J. Org. Chem. 53 (1988) 3583–3586.
[28] T. Masuda, M. Tomita, Y. Ishihama, Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis, J. Proteome Res. 7 (2008) 731–740.
[29] M. Iwasaki, T. Masuda, M. Tomita, Y. Ishihama, Chemical cleavage-assisted tryptic digestion for membrane proteome analysis, J. Proteome Res. 8 (2009) 3169–3175.