Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 3, 817–821

THERMODYNAMIC STUDIES OF THE BINDING INTERACTIONS OF SURFACTIN ANALOGUES TO LIPID VESICLES
Application of isothermal titration calorimetry

H. Razafindralambo[*]*, S. Dufour, M. Paquot and M. Deleu*

Unité de Chimie Biologique Industrielle, Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 Passage des Déportés 5030 Gembloux, Belgium

Isothermal titration calorimetry was applied for studying the binding interactions of cyclic and linear surfactins with different ionic charge (z=–2 and –3) and lipid chain length (n=14 and 18) to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-choline (POPC) vesicles in 10 mM Tris buffer at pH 8.5 with 150 mM NaCl at 25°C. Surfactin analogues interacted spontaneously ($\Delta G_D^{w\to b} < 0$) with POPC vesicles. The binding reactions were endothermic ($\Delta H_D^{w\to b} > 0$) and entropy-driven process ($\Delta S_D^{w\to b} > 0$). Moreover, significant differences in the binding constant values (K) ranging from $6.6\cdot10^3$ to $9.6\cdot10^4$ M^{-1} show that cyclic structure and the increase of lipid chain length are favourable on the surfactin binding affinity to POPC vesicles, whereas the rise of the number of negative charges has an opposite effect.

Keywords: *interaction, isothermal titration calorimetry, lipid vesicles, lipopeptide, surfactin*

Introduction

Surfactin is a class of lipopeptide compounds that receive wide-spread attention in scientific and industrial areas [1, 2]. This arises from the fundamental point of view of its particular amphiphilic structure such as the hybrid and intermediate features (i.e. combining small surfactant with lipid chain and protein structures, with middle molecular mass) providing it numerous excellent properties. Among these are the surfactant power [3], foaming and emulsifying properties [4–6], antiviral, antimycoplasma and antibacterial activities [7–9]. As a result, surfactin finds potential applications in agricultural, environmental, bio- technological, cosmetic and pharmaceutical fields.

Its basic structure consists of a heptapeptide linked to a β-hydroxy fatty acid, which represents those from natural and synthetic sources.

Natural surfactins are mainly excreted by various strains of *Bacillus subtilis*. Those biomolecules have a cyclic structure with a lactone bond between the hydroxyl group of the hydroxy fatty acid and the carboxy terminal group of the peptidic part [10]. The fatty acid chain length varies from 12 to 17 carbon atoms that constitute homologous series [11], whereas the heptapeptide primary structure differs in amino acid composition giving rise to isoform compounds [12].

Synthetic surfactins are cyclic [13, 14] or linear lipopeptides with different lipid chain lengths and ionisable acid residues [15]. Those compounds are mainly intended for studying structure-activity relationships and understanding mechanisms of action at molecular level.

Previously, it has been shown that small changes in molecular structure of surfactin affected its fundamental surface activities [16], foaming properties [17], haemolytic activity [15] and membrane-penetrating properties [18]. All these investigations provide not only fundamental information but they also enable the selection or design of the most efficient molecules for many applications. With regard to biological activities, it is generally assumed that surfactin properties depend on its interactions with the membrane of target cells. Thus, several investigations have been undertaken by using biophysical methods and various membrane models such as lipid monolayers [19, 20], bilayers [21, 22] and vesicles [23], or by computational approach [24, 25] in order to elucidate its mechanism of action.

In this work, isothermal titration calorimetry (ITC) was applied for studying, by thermodynamic approach, the role of the different structural entities of surfactin on its binding interactions to lipid vesicles. For this purpose, the effects of a natural cyclic and three synthetic linear surfactins with different peptide charges and lipid chain length were compared. During the last two years, ITC has been reported as a powerful tool to obtain relevant thermodynamic information on binding processes among (bio)molecules with various structures like proteins/peptides, surfactants, lipids and metal ions [26–28]. Therefore, it would appear as the most appropriate method for evaluating the binding affinity of surfactin analogues to spherical bilayer model membranes.

* Author for correspondence: razafindralambo.h@fsagx.ac.be

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Experimental

Materials

Chloroform and methanol were HPLC grades (Scharlau, Spain). Tris(hydroxymethyl)aminomethane (trizma) and NaCl were from Sigma (St-Louis). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was from Avanti Polar Lipids (Alabaster, AL).

Cyclic surfactin was extracted from a *Bacillus subtilis* S499 culture supernatant and purified as previously described [29]. Linear surfactin analogues were synthesized, purified and characterized according to Dufour *et al*. [15]. Their characteristics are listed in Table 1.

Preparation of lipid vesicles

A solution of POPC (7.6 mg in 5 mL) in chloroform/methanol (2/1, *v*/*v*) was dried and then put under vacuum overnight. The lipid film was hydrated in 5 mL buffer 10 mM Tris 150 mM pH 8.5 during 1 h at 37°C with vortex mixing applied every 15 min and then frozen in ice bath. The solution was sonicated for 25 min under 117 watts power using sonicator tip with 3 mm diameter (Vibracell 75185). At the end of experiment, the suspended vesicles were centrifuged in Eppendorf at 1000 g for 10 min.

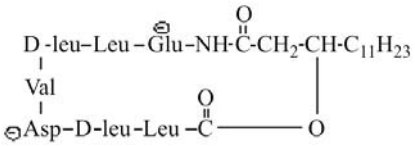
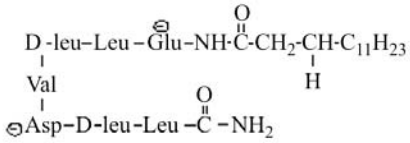
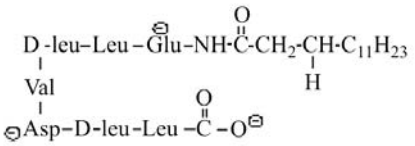
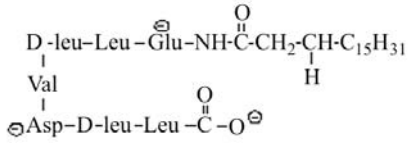
Methods

ITC experiments

ITC measurements were performed on a VP-ITC Microcalorimeter (Microcal, Northampton USA). Surfactin titrations were carried out by injecting by steps 1–20 μL aliquots of large unilamellar vesicles (LUVs) made with POPC (2 mM) into the calorimeter cell (V_{cell}=1.4565 mL) containing SNC14, SAL14, SSL14 or SSL18 10 μM at constant time intervals of 3–5 min and at 25°C.

All solutions were prepared with the same buffer constituted of 10 mM Tris, 150 mM NaCl pH 8.5 into milliQ water (Millipore Co., Milford, MA). The solution in the sample cell was stirred at a speed of 305 rpm. The reference cell was filled with buffer solution. Prior each analysis, all solutions were degassed using sonicator bath. The heats of dilution of surfactin solutions and vesicles were determined by injecting buffer in surfactin and vesicles in buffer. These values were substracted from the observed heats for determining effective heats. All measurements were repeated four times with two different vesicle preparations. Raw data were processed by software Origin 7 (Originlab, Northampton, USA).

Table 1 Characteristics of surfactin analogues

Surfactin abbreviation	Structure	*MM*/g mol^{-1}	Charge at pH 8.5, *z*	C atom number of alkyl chain, *n*
SNC14		1021	–2	14
SAL14		1022	–2	14
SSL14		1023	–3	14
SSL18		1079	–3	18

Lipid vesicle size measurement

The average size of POPC vesicle suspension was determined at 25°C by dynamic light scattering (DLS) method using a Zetasizer nano ZS (Malvern instruments, UK) with a He–Ne laser source at a wavelength 633 nm. The scattered light intensity was measured at a scattering angle of 173°.

Thermodynamic binding model and calculation

The ITC data were evaluated by using the model described by Heerklotz and Seelig [23]. The binding coefficient K is given by the equation

$$K = \frac{R_b}{C_{D,f}} \quad (1)$$

where R_b is the surfactant-to-lipid ratio (degree of binding) and $C_{D,f}$ the concentration of surfactin free in solution.

If n_L^0 and $n_{D,b}$ are referred to the molar amounts of total lipid in the calorimeter sample cell and bound surfactin, respectively, and $C_{D,b}$ and C_L^0 the concentrations of bound surfactin and lipid in the calorimeter cell, R_b can be rewritten as:

$$R_b = \frac{n_{D,b}}{n_L^0} = \frac{C_{D,b}}{C_L^0} \quad (2)$$

The total amount of surfactin in the cell, C_D^0, is represented by the following equation

$$C_D^0 = C_{D,b} + C_{D,f} \quad (3)$$

By combining Eqs (2) and (3), Eq. (1) can be written as

$$C_{D,b} = C_D^0 \frac{KC_L^0}{1 + KC_L^0} \quad (4)$$

After i^{th} lipid injections the molar amount of bound surfactin in the calorimeter cell is $n_{D,b}(i)$ and the cumulative heat absorbed is

$$\sum_{k=1}^{i} \delta h_k = n_{D,b}^{(i)} \Delta H_D^{w\to b} = \Delta H_D^{w\to b} C_{D,b}^{(i)} V_{cell} = \\ = \Delta H_D^{w\to b} V_{cell} C_D^0 \frac{KC_L^0}{1 + KC_L^0} \quad (5)$$

where $\Delta H_D^{w\to b}$ is the molar enthalpy change corresponding to the transfer of surfactin from the aqueous phase (w) to the bilayer membrane (b).

Thus K and $\Delta H_D^{w\to b}$ can be evaluated simultaneously by a fit of the measured cumulative heat as a function of C_L^0.

Results and discussion

Surfactin binding to lipid vesicles was thermodynamically characterized by lipid-into-surfactin titration. LUVs (~100 nm diameter, 2 mM) of POPC were injected in steps into the sample cell containing 10 μM of surfactin analogues. The heat flow was measured and recorded as a function of time. The reaction heats were obtained by integration of the titration peaks. Figure 1 shows the cumulative heats as a function of lipid concentration for surfactin analogues investigated in this study. By fitting the data according to Eq. (5), the binding constant K and heat of transfer of surfactin from aqueous phase to bilayer membrane, $\Delta H_D^{w\to b}$, were evaluated simultaneously. Corresponding free energy $\Delta G_D^{w\to b}$ and reaction entropy $\Delta S_D^{w\to b}$ were then calculated by the standard equations: $\Delta G = -RT\ln(KC_w) = \Delta H - T\Delta S$ with R=8.31 J mol^{-1} K^{-1} and C_w=55.5 M. Thermodynamic parameters for membrane binding of surfactin analogues at 25°C are listed in Table 2. In general, all surfactins have an af-

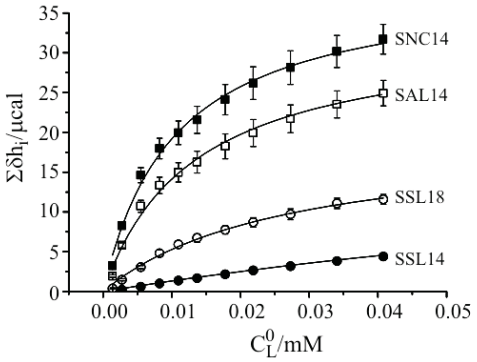


Fig. 1 Cumulative heats *vs.* total lipid concentration as obtained from surfactin analogues (10 μM) titration with POPC vesicles at 25°C. The solid lines correspond to theoretical fits of the total cumulative heat (average of four replicates) according to Eq. (5)

Table 2 Thermodynamic parameters for the binding of surfactin analogues to POPC vesicles at 25°C

Compound	K/mM^{-1}	$\Delta H_D^{w\to b}$/kJ mol^{-1}	$\Delta G_D^{w\to b}$/kJ mol^{-1}	$T\Delta S_D^{w\to b}$/kJ mol^{-1}
SNC14	96.3±5.3	11.2±0.2	–38.2±0.4	49.5±0.6
SAL14	75.8±4.8	9.4±0.2	–37.7±0.3	47.1±0.5
SSL14	6.6±0.3	6.2±0.2	–31.7±0.2	37.9±0.4
SSL18	37.5±3.6	5.6±0.3	–36.0±0.5	41.6±0.8

finity to POPC vesicles for which the binding reactions are spontaneous ($\Delta G<0$), endothermic ($\Delta H>0$), and generate a positive change of the system entropy ($\Delta S>0$) indicating entropy-driven processes. On the other hand, significant differences in K values ranging from $6.6\cdot10^3$ to $9.6\cdot10^4$ M^{-1} were observed among surfactin analogues that allow us to point out the effect of molecule structural attributes on its interaction with POPC vesicles.

Surfactin cycle effect

The effect of peptide cycle can be assessed by comparing SNC14, the cyclic surfactin, with SAL14 the linear one, which bring the same negative charge ($z=-2$) and lipid chain length ($n=14$). Based on K value, SNC14 exhibits higher affinity than SAL14 suggesting the positive effect of the peptide cycle on surfactin binding to lipid vesicles. However, ΔG, ΔH and ΔS values are quite similar indicating that the two compounds have the same effect in term of energy involved in the binding interaction.

Surfactin negative charge effect

At pH 8.5, surfactin carboxyl residues are totally ionised. Thus, by comparing SAL14 to SSL14 containing, respectively, 2 and 3 carboxyl residues, the effect of the negative charge and then the electrostatic interaction may be pointed out, the two compounds being constituted by linear lipopeptides structures. According to ΔH, ΔG and K values, the binding affinity of surfactin on lipid vesicles decreases with increasing the number of the negative charge in the peptide part. Significant changes were observed with the K values, which decrease from $7.6\cdot10^4$ to $6.6\cdot10^3$ M^{-1} suggesting that electrostatic strength also contributes to the binding mechanism of surfactin with the model membrane under study.

Surfactin lipid chain length effect

The effect of the lipid chain length was evaluated based on the thermodynamic data obtained with SSL14 and SSL18, which contain a fatty acid with 14 and 18 carbons, respectively. Both surfactins are constituted with linear peptide chain and bring three negative charges ($z=-3$). It clearly appears that the K value increases with increasing the lipid chain length, and ΔG becomes more negative indicating the increase of the degree of surfactin binding to POPC vesicles. These results demonstrate the important role of the hydrophobic effect on surfactin–lipid membrane interactions. The more hydrophobic the fatty acid associated with the peptide moiety, the higher its binding affinity with lipid membranes.

The present study aims at determining the structural effect of surfactin on its interaction with phospholipid vesicles by using ITC technique. For this purpose, the binding affinity of a natural cyclic and three linear surfactins with well-defined structures have been compared based on thermodynamic parameters. The values obtained in this work for SNC14, as reference, are quite similar to those reported by Heerklotz and Seelig [23], except the binding constant K which was slightly higher ($9.6\cdot10^4$ *vs.* $2.2\cdot10^4$ M^{-1}). This disparity could be explained by the difference in the ionic strength and the composition of the sample used. Indeed, a higher ionic strength of surfactin solution in our experiments (150 compared to 100 mM) probably reinforces the hydrophobic interactions and therefore increases the binding affinity, which depends on the K value. Moreover, the sample used by these authors was a mixture of surfactin homologous C13, C14 and C15 in different proportions, and not only constituted by unique surfactin as SNC14. The results of our ITC experiments show that surfactin interaction with POPC vesicles depends on both peptide and lipid structural characteristics such as the cycle, the number of negative ionic charges, and the lipid chain length. The favourable effects of the cyclic structure and the increase of lipid chain length on surfactin binding affinity to POPC vesicles are in accordance to their higher surface-active, monolayer stability, micelle capacity, and phospholipid monolayer penetrating properties, as reported previously [15, 18]. Evidence explanation arises from the conformational change of surfactin when the peptide is in the cyclic or linear form. Indeed, linear surfactin adopts an α-helical conformation when its concentration is below the critical micellar concentration [30] as it is the case in this study, whereas the cyclic surfactin exhibits 'horse saddle-like structure' favouring distinct polar and apolar domains [31]. This conformation reinforces the surfactin amphiphilic nature and then its affinity to any interfaces. Similarly, increasing lipid chain length strengthens the hydrophobic interaction, which is a common binding mechanism of amphiphilic peptides to bilayer phospholipids [32]. On the other hand, the unfavourable effect with increasing the peptide negative charge could be easily interpreted by a stronger electrostatic repulsion between free and membrane bound surfactin molecules, but also by a higher affinity in water, both lead to the decrease of the surfactin binding affinity to lipid vesicles.

Conclusions

In conclusion, the present investigation shows that ITC method is a sensitive technique for evaluating the structural change effect on the binding affinity of surfactin to phospholipid vesicles as model membrane. By thermodynamic approach, it appears that surfactin analogues spontaneously interact to POPC vesicles, the reaction being endothermic and entropy-driven process. The loss of cyclic structure and the increase of the global negative charge of the peptide part weaken the degree of surfactin binding, whereas the increase of the lipid chain length reinforces it. One can also deduce that both hydrophobic and electrostatic effects contribute to the surfactin binding affinity to zwitterionic phospholipid vesicles. This approach is very important for both fundamental and applied researches since the binding step constitutes undoubtedly a key factor in the membrane-active properties of bioactive molecules. Another important aspect to investigate by ITC technique would be the capacity of surfactin analogues to solubilise lipid vesicles.

Acknowledgements

M.D. is Research Associate of The National Foundation for Scientific Research (F.N.R.S.) from Belgium. This work received the financial support from the Walloon Region (convention 021/5103). The authors thank Prof. P. Thonart and his team (Unité de Bio-industries, Faculté Universitaire des Sciences Agronomiques de Gembloux, Belgium) for the production of the natural cyclic surfactin by fermentation and Mrs Edith Staes for the measurements of POPC vesicle size.

References

1 J. Desai and I. Banat, Mol. Biol. Rev., 61 (1997) 47.
2 D. Solaiman, Inform., 16 (2005) 409.
3 K. Arima, A. Kakinuma and G. Tamura, Biochem. Biophys. Res. Commun., 31 (1968) 488.
4 H. Razafindralambo, M. Paquot, A. Baniel, Y. Popineau, C. Hbid, P. Jacques and P. Thonart, J. Am. Oil Chem. Soc., 73 (1996) 149.
5 H. Razafindralambo, M. Paquot, A. Baniel, Y. Popineau, C. Hbid, P. Jacques and P. Thonart, Food Hydrocoll., 11 (1997) 59.
6 M. Deleu, H. Razafindralambo, Y. Popineau, P. Jacques, P. Thonart and M. Paquot, Colloids Surf., A Physicochem. Eng. Asp., 152 (1999) 3.
7 H. Itokawa, T. Miyashita, H. Morita, K. Takeya, T. Hirano, M. Homma and K. Oka, Chem. Pharm. Bull., 42 (1994) 604.
8 D. Vollenbroich, G. Pauli, M. Özel and J. Vater, Appl. Environ. Microbiol., 63 (1997) 44.
9 M. Kracht, H. Rokos, M. Özel, M. Kowall, G. Pauli and J. Vater, J. Antibiot. (Tokyo), 52 (1999) 613.
10 A. Kakinuma, A. Ouchida, T. Shima, H. Sugino, M. Isono, G. Tamura and K. Arima, Agric. Biol. Chem., 33 (1969) 1669.
11 X. Liu, N. Haddad, S. Yang and B. Mu, Protein Peptide Lett., 14 (2007) 766.
12 F. Peypoux, J. M. Bonmatin and J. Wallach, Appl. Microbiol. Biotechnol., 51 (1999) 553.
13 S. Nagai, K. Okimura, N. Kaizawa, K. Ohki and S. Kanamoto, Chem. Pharm. Bull., 44 (1996) 5.
14 M. Pagadoy, F. Peypoux and J. Wallach, Int. J. Pept. Res. Ther., 11 (2005) 195.
15 S. Dufour, M. Deleu, K. Nott, B. Wathelet, P. Thonart and M. Paquot, Biochim. Biophys. Acta Gen. Subj., 1726 (2005) 87.
16 H. Razafindralambo, P. Thonart and M. Paquot, J. Surfactants Deterg., 7 (2004) 41.
17 H. Razafindralambo, Y. Popineau, M. Deleu, C. Hbid, P. Jacques, P. Thonart and M. Paquot, J. Agric. Food Chem., 46 (1998) 911.
18 M. Eeman, A. Berquand, Y. F. Dufrêne, M. Paquot, S. Dufour and M. Deleu, Langmuir, 22 (2006) 11337.
19 R. Maget-Dana and M. Ptak, Biophys. J., 68 (1995) 1037.
20 M. Deleu, M. Paquot, P. Jacques, P. Thonart, Y. Adriaensen and Y. F. Dufrêne, Biophys. J., 77 (1999) 2304.
21 D. Sheppard, C. Jumarie, D. G. Cooper and R. Laprade, Biochim. Biophys. Acta, 1064 (1991) 13.
22 R. Brasseur, N. Braun, K. El Kirat, M. Deleu, M.-P. Mingeot-Leclercq and Y. F. Dufrêne, Langmuir, 23 (2007) 9769.
23 H. Heerklotz and J. Seelig, Biophys. J., 81 (2001) 1547.
24 M. Deleu, O. Bouffioux, H. Razafindralambo, M. Paquot, C. Hbid, P. Thonart, P. Jacques and R. Brasseur, Langmuir, 19 (2003) 3377.
25 O. Bouffioux, A. Berquand, M. Eeman, M. Paquot, Y. F. Dufrêne, R. Brasseur and M. Deleu, Biochim. Biophys. Acta, 1768 (2007) 1758.
26 A. Ziegler, Adv. Drug Delivery Rev., 60 (2008) 580.
27 A. Saboury, M. Atri, M. Sanati and M. Saghadi, J. Therm. Anal. Cal., 83 (2006) 175.
28 A. Bordbar, R. Hosseinzadeh and M. Norozi, J. Therm. Anal. Cal., 87 (2007) 453.
29 H. Razafindralambo, M. Paquot, C. Hbid, P. Jacques, J. Destain and P. Thonart, J. Chromatogr. A, 639 (1993) 81.
30 M. Osman, Y. Ishigami, K. Ishikawa, Y. Ishizuka and H. Holmsen, Biotechnol. Lett., 16 (1994) 913.
31 J. Bonmatin, M. Genest, H. Labbé and M. Ptak, Biopolymers, 37 (1994) 975.
32 J. Seelig, Biochim. Biophys. Acta, 1331 (1997) 103.

DOI: 10.1007/s10973-008-9403-6