Effect of Functional Groups on Incorporation Behavior of Amino Acid-Type Surfactant into Phospholipid Vesicle Membrane

Akio Ohta^{1*}, Yuya Miyazato¹, Hiroyasu Sasaki¹, Kazuma Yasuhara² and Tsuyoshi Asakawa¹

¹ School of Chemistry, College of Science and Engineering, Kanazawa University (Kakuma, Kanazawa, Ishikawa 920-1192, JAPAN)

² Graduate School of Material Science, Nara Institute of Science and Technology (8916-5, Takayama-cho, Ikoma, Nara 630-0192, JAPAN)

Abstract: The incorporation behaviors of some *N*-dodecanoyl amino acid-type surfactants into *L*- α -dipalmitoylphosphatidylcholine (DPPC) vesicles in aqueous solution were investigated. From the leakage measurements of a vesicle-entrapped fluorescence probe, it was found that these surfactants did not affect the DPPC vesicle so much at a very low concentration less than a one-tenth of the critical micelle concentration (CMC), but caused a significant release of the probe from the vesicles even at just below the CMC. The leakage induced by amino acid-type surfactants was promoted by increasing hydrophobicity of the amino acid. However the polarization of DPH embedded in the DPPC membrane was almost unchanged by incorporation of the surfactants at the concentration below the CMC except for the *N*-dodecanoylphenylalanine system. The binding enthalpy and the binding constant of the surfactant to DPPC vesicles were estimated by isothermal titration calorimetry. While the binding enthalpy was independent of the kind of surfactant, the binding constant increases with increasing hydrophobicity of the amino acid species in the surfactants. The molar ratio of bound surfactant to DPPC molecule in the saturated state was much greater than that for sodium dodecyl sulfate system. Further the ratio was not influenced by the structure of the amino acid side chain.

Key words: *N*-acyl amino acid-type surfactant, phospholipid, vesicle, fluorescence probe, ITC

1 INTRODUCTION

Phospholipid is the main component of biological cell membrane. The interaction between phospholipid vesicle membrane and surfactant is of great interest from the standpoints of the solubilization and the reconstitution of membrane proteins, and hence, many researchers have reviewed about it¹⁻⁶. In general, the nonionic surfactants have been widely used for this purpose⁷⁻¹⁷. On the other hand, anionic surfactants are used as detergents, and then the interaction of anionic surfactants with phospholipid vesicles might be a good research model for the influence of detergent on skin¹⁸⁻²¹. Particularly *N*-acyl amino acids, which are anionic amino acid-type surfactants, are very valuable because of their biodegradability and low toxicity^{22,23}. In spite of the interest in their low toxicity, there are few studies on

the interaction between amino acid-type surfactants and phospholipid vesicles from the standpoint of their physicochemical properties, except for those concerning their antimicrobial activity²⁴⁻²⁶.

In our previous study, the incorporation behaviors of sodium *N*-dodecanoyl-sarcosinate and *L*-glutamate into phospholipid vesicle were examined, and the results were compared with those of sodium dodecyl sulfate (SDS) and sodium dodecanesulfonate²⁷. This study revealed the unique behaviors of amino acid-type surfactants; i.e., the cooperative adsorption on the vesicle membrane occurs for amino acid-type surfactant systems, and these surfactant molecules are organized on the vesicle. Therefore, in this work, the incorporation behavior of some amino acid-type surfactants into DPPC vesicles was investigated systematically for the purpose of an elucidation of the effect of amino acid side chain. We chose the following four amino

*Correspondence to: Akio Ohta, School of Chemistry, College of Science and Engineering, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, JAPAN

E-mail: akio-o@t.kanazawa-u.ac.jp

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acid surfactants, sodium *N*-dodecanoyl-glycinate (C12Gly), -L-valinate (C12Val), -L-leucinate (C12Leu), and -L-phenylalaninate (C12Phe).

It is known that the critical micelle concentration (CMC) of the amino acid surfactants decreases monotonously with increasing hydrophobicity of the amino acid side chain without exception²⁸. The negative enthalpy of micelle formation and high Krafft temperature is characteristic of C12Phe. Although these phenomena could not be explained in terms of only the hydrophobicity of the side chain^{28,29}, these results suggest the presence of a strong interaction between the side chains of phenylalanine in the micelle or hydrated solid states. In such a way, the side chain of *N*-acyl amino acid surfactants greatly influences their physical properties. Thus, it is important to investigate the effect of the amino acid side chain on the incorporation behavior of these surfactants into DPPC vesicle membranes. This was done mainly by the fluorescence measurements for both the release of a vesicle-entrapped probe and the polarization of DPH in lipid membrane in the presence of a series of surfactant as a function of the surfactant concentration. In addition, the binding constant and the binding enthalpy of the surfactant to DPPC vesicles were estimated by isothermal titration calorimetry (ITC).

2 EXPERIMENTAL

2.1 Materials

Sodium *N*-dodecanoyl glycinate (C12Gly), sodium *N*-dodecanoyl L-valinate (C12Val), sodium *N*-dodecanoyl L-leucinate (C12Leu), and sodium *N*-dodecanoyl L-phenylalaninate (C12Phe) were prepared from respective *N*-dodecanoyl amino acids by addition of an equivalent quantity of sodium hydroxide. All the above substances were purified by recrystallization from ethanol solution. Except for C12Gly, L-amino acids were employed as an ingredient. DPPC and 5(6)-carboxy fluorescein (CF) were purchased from Wako Pure Chemical Industries Ltd. and Kanto Chemical Co. Inc., respectively, and were used as received. In this experiment, CF was used as a mono-sodium salt. For the preparation of vesicular suspensions, 0.1 M tris(hydroxymethyl)-aminomethane (tris)-HCl buffer at pH = 7.4 was employed. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorescence probe for the vesicle membrane, which was purchased from Aldrich.

Vesicle suspensions were prepared by hydration of a dry DPPC film (20 mg) with 2 mL of tris-HCl buffer or that with CF (0.1 M). Finally, the vesicle suspensions were extruded into 100 nm polycarbonate filter on an extruder (Avanti Polar Lipids Mini-Extruder) at 320 K³⁰. The CF encapsulating vesicles were separated from untrapped CF molecules by gel filtration. The concentration of DPPC was confirmed

by determining the quantity of the choline group using a special assay kit from Wako Pure Chemicals³¹.

2.2 Surface tension measurements

The surface tension of the surfactant solution was measured at 298.2 K using the Wilhelmy glass plate method with a Kyowa EBS-V tensiometer (Saitama, Japan) with an accuracy of $\pm 0.2 \text{ mNm}^{-1}$.

2.3 Leakage Measurements

About ten microliters of vesicle suspension were added to 1 mL portions of several surfactant solutions so that the concentration of DPPC was 0.1 mM. Fluorescence measurements were performed using a Hitachi F-3010 fluorescence spectrometer at 298.2 K. The fluorescence intensity of leaked CF was measured at 515 nm by excitation at 490 nm. Excitation and emission band-passes were 1.5 nm. At the end of the measurements, 100 μL of 10 wt% Triton X-100 solution was added to all fractions in order to rupture all of the vesicles, and the fluorescence intensity was measured again.

2.4 Polarization measurements

The polarization P defined by

$$P = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}} \quad (1)$$

was obtained from the fluorescence intensity of DPH by using a Perkin Elmer LS55 spectrometer. Here G is the grating factor, which is calculated by the following equation

$$G = I_{hv} / I_{hh}, \quad (2)$$

where I_{hv} is the intensity with horizontal and vertical polarizers (excitation and emission, respectively), and I_{hh} is the intensity with horizontal polarizers for both emission and excitation. The fluorescence intensity of DPH was measured at 428 nm by excitation at 357 nm. It has been reported that DPH is oriented to the lipid membrane³². Therefore, the molecular motion is restricted at high viscosity and the P value reflects the fluidity at the hydrophobic core of the molecular aggregates. DPH molecules were added to methanol solution of DPPC, and then dry DPPC film with DPH was prepared. The ratio of DPPC to DPH was 1000 : 1 and the final concentration of DPH was 10^{-7} M, whereas DPPC concentration in each sample was 0.1 mM.

2.5 Calorimetry

The enthalpy of dilution of a concentrated solution of the surfactant was measured by isothermal titration microcalorimetry (MCS-ITC, MicroCal, Northampton, MA) at 298.15 K. Ten microliters of the aqueous solution of each

surfactant at ca. 15-fold concentration of their CMC was injected using a computer-controlled syringe pump into 1.5 mL of tris-HCl buffer solution or DPPC suspension in the cell. DPPC concentration was varied from 0.02 to 0.14 mM.

3 RESULTS and DISCUSSION

3.1 Critical Micelle Concentrations by Surface Tension Measurements

The surface tension γ of the aqueous solutions of several surfactants was shown in Fig.1 as a function of molarity c_s of the surfactant at 298.2 K. The γ values of the aqueous solution in the presence of 0.1 mM DPPC were over-plotted in respective figures. The γ values decrease steeply with increasing c_s and all curves have a break point at CMC. It is seen that the γ values of the solutions of C12Gly and C12Leu are not changed even though the DPPC vesicles coexist. In contrast, the γ value for C12Phe system significantly increased in the range of 0.1 - 0.6 mM by the addition of DPPC vesicles following increase of CMC. Furthermore we observed another break point at lower concentration in the curve of C12Phe with DPPC vesicle system. It is suggested that, in the region between two breakpoints, the C12Phe molecules are highly accumulated on the DPPC vesicle membrane below the CMC, thus, its concentration in bulk phase reduced. On the other hand, both surface tension of aqueous solution and the CMC values of SDS slightly decreased in the presence of DPPC vesicles. This fact suggests that SDS molecules affect the DPPC vesicles below the CMC to induce the spontaneous formation of mixed monolayer of SDS and DPPC at the air/water interface³³⁾.

3.2 Leakage and Fluidity of Vesicle Membrane

The values of the leakage ratio of CF molecules, which

were estimated from the fluorescence intensity of the samples, were plotted in Fig.2 as a function of time for the four amino acid-type surfactants at various concentrations, $C^* (= c_s / \text{CMC} : \text{CMC is obtained by surface tension measurement with DPPC})$. The leakage ratio L was defined by

$$L(\%) = \frac{I - I_{\text{bg}}}{I_{\text{triton}} \times r - I_{\text{bg}}} \times 100 \quad [3]$$

where I and I_{triton} are the fluorescence intensity of the samples during the measurements and at the end of the measurements when all vesicles were completely ruptured by Triton X-100, respectively, and r is the dilution factor due to the addition of 100 μL of 10 wt% Triton X-100 solution; in this case, the r value was 1.1. The background intensity I_{bg} was estimated by

$$I_{\text{bg}} = I_{\text{triton}} \times \frac{I_{\text{buffer}}}{I_{\text{triton+buffer}}} \quad [4]$$

where I_{buffer} is the fluorescence intensity of the surfactant-free sample and $I_{\text{triton+buffer}}$ is that of the mixture of the sample and Triton X-100. For all the systems, the leakage of CF from the vesicles was hardly induced by the addition of the surfactants below $C^* = 0.02$ (Fig.2(a)). On the other hand, above $C^* = 1$ the encapsulated CF was released spontaneously except for C12Gly system. At intermediate concentration, $C^* = 0.2$ (Fig.2(b)), the leakage kinetics are different depending on the type of surfactants. The initial rate of dye leakage increased with increasing the hydrophobicity of amino acid.

The values of the leakage ratio at 2 h after the mixing were also plotted against the reduced concentration C^* in Fig.3 for the four surfactant systems. Above $C^* = 1$, the release of CF molecules was completed for all systems. This result shows that these surfactant molecules ruptured the DPPC vesicles to form the mixed micelles. However the encapsulated CF in DPPC vesicles was already

Fig.1 Surface Tension vs Concentration Curves for Various Surfactants in 0.1M tris-HCl Buffer Solution at 298.2 K. Open circles: with 0.1mM of DPPC, filled circles: without vesicle. (a) SDS, (b) C12Gly, (c) C12Leu, (d) C12Phe.

Fig.2 Leakage Ratio of CF vs Time for DPPC Vesicles in Each Surfactant Solution.

The reduced surfactant concentration, $C^* = c_s/\text{CMC}$, is (a) 0.02, (b) 0.2, (c) 1 and (d) 4.

released mostly below the CMC for all systems. In particular for the hydrophobic amino acid systems, the leakage ratio reached 100 % even at the concentration lower than the CMC. These results suggest that the solubilization of liposome is not the only mechanism of dye leakage induced by amino acid surfactants. In the previous study, we have reported that several amino acid type surfactants interact with DPPC vesicles cooperatively to form pore structure in the vesicle membrane, which is large enough for CF molecules to pass through the membrane^{27,34}. Karatekin et al. have reported the toroidal membrane pore formed by surfactant molecules^{35, 36}. The CF molecules might pass through the pore consisting of amino acid type surfactants.

Next, we examined the effect of surfactants on membrane fluidity by measuring the polarization of DPH embedded in DPPC membrane. The polarization in the presence of surfactant, P , was divided by that in surfactant-free sample, P_0 ($= 0.42 \pm 0.03$) to obtain the relative polarization, P/P_0 . Figure 4 shows the surfactant concentration dependence of P/P_0 values measured at 2 h after the addition of surfactant. The P values did not change with time in contrast to the dye leakage (Data not shown). It is seen that below the CMCs, the P/P_0 values hardly change from that in surfactant-free solution regardless of a variation of the surfactant concentration except for the C12Phe system. It is interesting that the P/P_0 values around $C^* = 0.5$ are still kept at unity whereas the encapsu-

Fig.3 CF Leakage Ratio vs Reduced Surfactant Concentration Curves for DPPC Vesicles in Each Surfactant Solution at 298.2 K.

Crosses: just after mixing, filled circles: at two hours after mixing.

(a) C12Gly, (b) C12Val, (c) C12Leu, (d) C12Phe.

Fig.4 Polarization Ratio vs Reduced Surfactant Concentration Curves for DPPC Vesicle Membrane in Each Surfactant Solution Obtained at Two Hours after Mixing at 298.2 K.

Inserted arrow indicates $C^* = 0.5$.

lated probe molecules in these vesicles are almost released under the same condition. This fact supports the above idea that these amino acid-type surfactants are localized to oligomeric domain structure. Therefore, it is hard for the probe molecules to interact with surfactant molecules in lipid membrane. Above the CMC, the P/P_0 values steeply decreased because of the solubilization of probe molecules

into mixed micelles of DPPC and surfactant, which have much higher fluidity than vesicles. It is noted that the P/P_0 value of C12Phe system exhibits a two-step decrease with increasing concentration. This unique trend was also observed in the surface tension measurement as described above. This result suggests that C12Phe-DPPC complex forms a different phase just below the CMC. Although the structure of this phase is not clear at the present time, the lipid membrane in this phase displays higher fluidity and the significant leakage of CF in this concentration region.

3.3 Calorimetry

In order to clarify the interaction between DPPC vesicle and the amino acid surfactants, the heat of titration of their micellar solutions to some vesicle suspensions of different concentrations was measured by ITC at 298.15 K. The differential enthalpy Δh_s , which was obtained by dividing the titration heat by the number of moles of injected solute, was plotted against the concentration of surfactant (Fig.5). The result of sodium dodecyl sulfate (SDS) system was shown in Fig.5(a) for comparison. The calorimetric examination for C12Val system was omitted in this study, since it is expected from the fluorescence results that C12Val system bears a striking resemblance to C12Leu system.

Typical sigmoidal curves were obtained for all systems without DPPC, and then we obtained the value of enthalpy of micelle formation from these curves²⁸⁾. The values of enthalpy of micelle formation are positive for C12Gly and C12Leu systems, while they are negative for C12Phe and SDS systems. It has been found that the dehydration of the surfactant usually plays an important role in micelle formation of *N*-acyl amino acid surfactant²⁸⁾, which contributes to positive enthalpy change. For the case of micelle forma-

tion of C12Phe, however, the attractive interaction between the benzyl groups of side chain of C12Phe would bring about a decrease of the enthalpy change upon micellization, resulting in the negative enthalpy of micelle formation. It is seen that the differential enthalpy value does not depend on the DPPC concentration so much at lower surfactant concentration for the amino-acid surfactant systems. For the SDS system, on the other hand, the enthalpy values deviated from the curve for the DPPC-free solution at very low concentration. This result supports our previous finding that the SDS molecules were adsorbed homogeneously on DPPC vesicles even at very low concentrations below $C^*=0.1$, while the amino acid surfactants hardly affect the property of membrane in the low concentration range²⁷⁾. However when the surfactant concentration approached to the CMC, the endothermic peak appeared on the differential enthalpy vs concentration curve, and then the enthalpy value of the peak top increased with increasing DPPC concentration for all surfactant systems. This implies that the large enthalpy is necessary for the incorporation of surfactant into the vesicles. It is suggested, furthermore, that the incorporation of surfactant reaches maximum at the peak top concentration, and the mixed micelles begin to form beyond the concentration. Therefore the concentrations at the peak top of these curves were considered as the CMC of the surfactants in the presence of DPPC vesicles. These results are consistent with those obtained by the surface tension measurements. That is, the addition of the DPPC vesicles brought about an increase of CMC in C12Phe system and a decrease in SDS system, and did not give a significant effect on the CMCs of C12Gly and C12Leu.

If we can neglect the changes in concentrations of solvent and DPPC during the titration, the differential

Fig.5 Titration Heat per Mole of Surfactant vs Concentration Curves with/without DPPC at 298.15 K.

Open circles : $c_L = 0.023$ mM, filled circles: $c_L = 0.046$ mM, diamonds: $c_L = 0.092$ mM, crosses: $c_L = 0.138$ mM, solid line: without DPPC.

(a) SDS, (b) C12Gly, (c) C12Leu, (d) C12Phe.

enthalpy Δh_s is regarded as the partial derivative of enthalpy of mixing H^M with respect to moles of surfactant n_s , and is expressed as following equation,

$$\Delta h_s \approx \left(\partial H^M / \partial n_s \right)_{T,p,n_i \neq s} = h_s - h_s^* \quad [5]$$

where h_s is the partial molar enthalpy of surfactant in the solution after mixing and h_s^* is that in the titrant solution of surfactant, respectively. Now let us consider the two states for the surfactant molecules; one is the free monomer in solution and the other is the bound one to the DPPC vesicles. Then eq.[5] is changed as

$$\Delta h_s \approx \left(\partial c_s^f / \partial c_s \right)_{T,p} (h_s^f - h_s^*) + \left(\partial c_s^b / \partial c_s \right)_{T,p} (h_s^b - h_s^*) \quad [6]$$

Here c_s is molarity of surfactant, and the superscripts “f” and “b” refer to the free monomer state in solution and the bound state to the DPPC vesicles, respectively. The total molarity of the surfactant is given by the mass balance relation:

$$c_s = c_s^f + c_s^b. \quad [7]$$

Next we consider the chemical equilibrium between the surfactant monomer in solution and that bound to vesicles. The binding constant of surfactant to vesicles is defined by

$$K = \frac{c_s^b}{c_L v_L c_s^f}. \quad [8]$$

Here c_L and v_L are molarity and molar volume of DPPC. Substitution of eqs. [7] and [8] into eq. [6] yields the expression:

$$\Delta h_s = \frac{K v_L c_L}{1 + K v_L c_L} \Delta h_s^b + \Delta h_s^f, \quad [9]$$

where the binding enthalpy of surfactant Δh_s^b and the enthalpy of dilution of surfactant Δh_s^f are defined as

$$\Delta h_s^b = h_s^b - h_s^f \quad [10]$$

and

$$\Delta h_s^f = h_s^f - h_s^*, \quad [11]$$

respectively. It was assumed that the molar volume of DPPC was independent of surfactant concentration.

The maximum enthalpy values of each plot in Fig.5 were plotted against c_L in Fig.6 for all surfactant systems, and eq. [9] was applied to data points for further analysis. The Δh_s^f values, which are roughly equal to the enthalpy of demicellization, can be obtained by the titration of surfactant solution without vesicles. Thus, the fitting of the experimental data as a function of c_L to Eq. [9] derives both the binding constant and the binding enthalpy. It is seen that the fitting curves reproduce the experimental results very well. Here v_L was estimated as $0.91 \text{ dm}^3 \text{ mol}^{-1}$ by the use of experimental data of molecular area $a_L (= 0.41 \text{ nm}^2)$ and bilayer pitch $d_L (= 7.4 \text{ nm})$ of hydrated DPPC in gel phase^{37,38}. Obtained results were summarized in Table 1. It is seen that the Δh_s^b value is positive and is hardly affected by a type of surfactant. The increase of enthalpy would be mainly due to a decrease of interaction between acyl chains of DPPC molecules in the vesicle membrane, which is caused by the insertion of the surfactant hydrocarbon chains into the membrane. This interpretation is consistent with the fact that the binding enthalpy is independent of the surfactant head group, but is determined by a hydrocarbon part of the surfactants.

Fig.6 Maximum Enthalpy vs DPPC Concentration Curves for Each Surfactant System. Solid lines are the fitting results by eq.[9].

Table 1 Calculated Thermodynamic Parameters by ITC.

surfactant	$K \times 10^3$	R	$\Delta h_s^b / \text{kJ mol}^{-1}$	$\Delta h_s^f / \text{kJ mol}^{-1}$	c_s^f / mM
C12Gly	2.3 ± 1.1	4.6	13.5 ± 3.0	-0.88	2.2
C12Leu	12 ± 0.1	3.1	13.4 ± 0.1	-4.0	0.28
C12Phe	28 ± 0.2	2.9	12.0 ± 0.4	3.6	0.12
SDS	2.7 ± 1.5	1.0	12.0 ± 5.0	2.0	0.43

On the other hand, the binding constant depends strongly on a kind of surfactant. Especially the amino acid surfactant with a hydrophobic side chain has a large value of K . This result suggests that an increase of entropy strongly dominates the binding process. Moreover, the hydrophobic effect of amino acid side chain is also one of the key factors for the binding of surfactant to lipid membrane.

To examine the binding of the surfactants to DPPC vesicles in more detail, we estimated the surfactant to DPPC molar ratio, R , in the vesicle membrane saturated with the surfactant molecules as follows:

$$R = \frac{c_s^b}{c_L} = K v_L c_s^f. \quad [12]$$

In this study, the concentration of the free monomer of surfactant c_s^f was calculated by eqs. [7] and [8] under the condition of $c_L = 0.046$ mM. Total concentration c_s in eq. [7] was considered as the concentration at which the differential enthalpy Δh_s was maximum in Fig. 5. Obtained data was also shown in Table 1. It is seen that the R values of these amino acid surfactant systems are greater than unity and increase with decreasing size of amino acid side chain. Although a number of surfactant molecules bound to the DPPC vesicle membrane, the environment around DPPC in the vesicle was not changed so much by the adsorption as demonstrated by polarization result. This result supports our depiction, in which the amino acid-type surfactant molecules are organized in the DPPC vesicle membrane^{27,34)}. On the other hand, R value of SDS system is almost unity, though SDS molecules affect the DPPC vesicle at even lower reduced concentration compared with the amino acid-type surfactant systems. It was confirmed again that this surfactant adsorbed homogeneously on DPPC vesicles.

4 CONCLUSION

The incorporation behaviors of four *N*-dodecanoyl amino acid-type surfactants to DPPC membrane in aqueous solution were investigated by fluorescence probe method and ITC. These surfactants did not leak the encapsulated probes in the DPPC vesicle so much in a low concentration range, but caused an immediate release of the probe from the vesicles at the concentration around the CMC. The leakage induced by amino acid-type surfactant was promoted by increasing hydrophobicity of the amino acid. The encapsulated probe molecules were released completely by incorporation of these surfactants even at the concentration below the CMC, whereas the polarization of the DPPC membrane was still high at the corresponding concentration. In addition, in both the surface tension and DPH measurement, C12Phe displayed different trend from other surfactants. The binding enthalpy and the binding constant of the surfactant to DPPC vesicles were

estimated by analyzing the ITC results thermodynamically. The binding enthalpy was almost independent of a type of surfactants; on the other hand, the binding constant increases with increasing hydrophobicity of the surfactant. The molar ratio of adsorbed surfactant to DPPC molecule in the saturated state for amino acid-type surfactant was much greater than that for sodium dodecyl sulfate system. The difference in the incorporation behavior between the amino acid-type surfactants and SDS can be discussed from the viewpoint of cooperative adsorption. That is, the amino acid-type surfactants might be incorporated into the DPPC vesicle cooperatively, and then, be organized on the membrane.

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