

Membrane fusion: what may transpire at the atomic level

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Target membrane proteins, SNAP-25 and syntaxin (t-SNARE), and secretory vesicle-associated membrane protein (v-SNARE), are part of the conserved protein complex involved in fusion of opposing bilayers in biological systems in the presence of calcium. It is known that SNARE interaction allows opposing bilayers to come close within a distance of approximately 3 Å, enabling calcium to drive membrane fusion. To understand the possible mechanism of membrane fusion at the atomic level, X-ray diffraction studies and light scattering measurements were performed in SNARE-reconstituted liposomes. Our study demonstrates that if t-SNARE vesicles and v-SNARE vesicles are allowed to interact prior to calcium addition, membrane fusion fails to occur. These studies suggest that hydrated calcium ions are too large (~6 Å) to fit between the space in SNARE-apposed bilayers, and therefore unable to induce membrane fusion. However in the presence of calcium, t-SNARE vesicles interact with v-SNARE vesicles, allowing the formation of calcium-phosphate bridges between the opposing bilayers, resulting in the expulsion of water due to disruption of the water shell around the calcium ion, enabling lipid mixing and membrane fusion.

Keywords: calcium bridges, hydrated calcium, membrane fusion, SNAREs

1. INTRODUCTION

The calcium ion is essential for life processes and is found in every cell. Ca^{2+} participates in diverse cellular processes, such as metabolism, secretion, proliferation, muscle contraction, cell adhesion, learning and memory. Although calcium is abundant within the cell, it is well sequestered and available on demand. Under the action of certain cellular stimuli, Ca^{2+} concentration at specific locations within the cell (nano environments) is elevated by several orders of magnitude within a brief period (< 1 ms in some cases). This prompt mobilization of Ca^{2+} is essential for many physiological processes, such as the release of neurotransmitters or cell signalling. A unique set of chemical and physical properties of the Ca^{2+} ion makes it ideal for participating in these biochemical reactions.

The calcium ion [Ca^{2+}] exists in its hydrated state within cells. The properties of hydrated calcium have been extensively studied using X-ray diffraction and neutron scattering in combination with molecular dynamics simulations [1–4]. The molecular dynamics simulations include three-body corrections compared with *ab initio* quantum mechanics/molecular mechanics molecular dynamics simulations. First principles molecular dynamics has also been used to investigate the structural, vibrational, and energetic properties of [$\text{Ca}(\text{H}_2\text{O})_n$] $^{2+}$ clusters and the hydration shell of the calcium ion. These studies demonstrate that hydrated calcium [$\text{Ca}(\text{H}_2\text{O})_n$] $^{2+}$

has more than one shell around it, and the first hydration shell around the Ca^{2+} has six water molecules in an octahedral arrangement [2]. In an earlier study [5], we reported that Ca^{2+} drives SNARE-induced fusion of opposing bilayers. In that study, SNARE interaction allow opposing bilayers to come close within a distance of approximately 3 Å. However, what transpired at the atomic level in that membrane fusion reaction was unclear. In the present study, using light scattering and X-ray diffraction experiments involving SNARE-reconstituted liposomes, we learn that fusion proceeds only when Ca^{2+} ions are available between the t- and v-SNARE-apposed bilayers. It has been demonstrated that t-SNAREs and v-SNARE in opposing bilayers interact in a circular array to form conducting pores in the presence of calcium [6]. This would suggest that Ca^{2+} ions need to be present between the SNARE-apposed bilayers, to bridge the opposing membranes. Once calcium forms such a bridge, it no longer can hold its water shells, leading to water expulsion, membrane destabilization and fusion.

2. MATERIAL AND METHODS

2.1. Preparation of liposomes

Lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama). A 10 mM lipid stock solution was prepared by mixing DOPC (1,2-dioleoyl phosphatidylcholine) and DOPS (1,2-dioleoyl phosphatidylserine) solutions in chloroform in a 70:30 mol/mol ratio in glass test tubes. The lipid mixture was dried under a gentle stream of nitrogen and resuspended in decane. The lipids

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were suspended in buffer containing 10 mM HEPES-NaOH (pH = 7.5) and 140 mM NaCl by vortexing for 5 min at room temperature. Large unilamellar vesicles (LUV) were formed following sonication for 2 min. Typically, vesicles in the diameter range 0.2–2 μm were obtained, as assessed by light and atomic force microscopies. Additionally two sets of proteoliposomes were prepared by gently mixing t-SNARE complex (Syntaxin-1/SNAP-25; final concentration 5 $\mu\text{g/ml}$) or VAMP2-His₆ (final concentration 2.5 $\mu\text{g/ml}$) with the liposomes [4, 6], followed by three freeze/thaw cycles to enhance protein reconstitution at the vesicle membrane.

2.2. Wide-angle X-ray diffraction studies

Ten microlitres of 10 mM t- and v-SNARE reconstituted liposomes in the presence or absence of Ca^{2+} were placed at the centre of an X-ray polycarbonate film, mounted on an aluminium sample holder and placed in a Rigaku RU2000 rotating anode X-ray diffractometer equipped with an automatic data collection unit (DATASCAN) and processing software (JADE). Experiments were performed at 25 °C. Samples were scanned with a rotating anode using the nickel-filtered $\text{Cu K}\alpha$ line ($\lambda = 1.5418 \text{ \AA}$) operating at 40 kV and 150 mA. Diffraction patterns were recorded digitally with a scan rate of 3°/min using a scintillation counter detector. The scattered X-ray intensities were evaluated as a function of scattering angle 2θ and converted into \AA using the formula $d = \lambda / 2 \sin\theta$.

2.3. Light scattering measurements

Vesicle aggregation and fusion were monitored using right angle (90°) light scattering with excitation and emission wavelengths set at 600 nm in a Hitachi F-2000 spectrophotometer [7]. Seven microlitres of t- and v-SNARE vesicles (at a final lipid concentration of 100 μM) were injected into a magnetically stirred cuvette containing 700 μl of assay buffer (in mM: HEPES 10 mM, NaCl 140 mM; pH = 7.4) at 37 °C. In experiments with Ca^{2+} , CaCl_2 (5 mM final concentration) was included in the assay buffer either prior to the addition of vesicles or added 4 min after the injection of vesicles. Light scattering intensities are expressed in arbitrary units. When vesicles fuse, there is less scattering of light.

3. RESULTS AND DISCUSSION

To monitor interaction(s) between Ca^{2+} ions and phosphate at the lipid membrane head groups, we employed our recently published X-ray diffraction method [5]. This experimental approach for monitoring interbilayer contacts requires the presence of (I) highly

concentrated lipid suspensions (10 mM and above) favouring multiple intervesicular contacts; and (II) fully hydrated liposomes, where vesicles have full freedom to interact with each other in solution, thereby establishing confined hydrated areas between adjacent bilayers [5]. This small fluid space could arise from interbilayer hydrogen bond formation through the water molecules [8], and additional bridging forces contributed due to the formation of trans-SNARE complexes [5, 6]. If these two conditions are met, then liposomes diffract as shown in Fig 1. Mixing of t- and v-SNARE liposomes in the absence of Ca^{2+} led to a diffuse and asymmetric diffractogram (depicted by the grey trace in Fig. 1), typically characteristic of short range ordering in a liquid system. In contrast, mixing of the t-SNARE and v-SNARE liposomes in the presence of Ca^{2+} led to a more structured diffractogram (depicted by the black trace in Fig. 1) with an approximately 12% increase in X-ray scattering intensity, reflected by an increase in the number of contacts between apposing bilayers established presumably by calcium-phosphate bridges, as previously suggested [9]. The ordering effect of Ca^{2+} on interbilayer contacts obtained in our X-ray studies is in good agreement with recent light and atomic force microscopy and spectroscopic studies suggesting close apposition of phosphate lipid head groups in the presence of Ca^{2+} ions followed by the formation of Ca^{2+} -phosphate bridges between adjacent bilayers [5,10]. Our X-ray studies show that the effect of Ca^{2+} on bilayer orientation and interbilayer contacts was most prominent around 3 \AA , with the appearance of an additional peak (shoulder) position at 2.8 \AA (depicted by the arrowhead in Fig. 1), both within the ionic radius of Ca^{2+} . These results suggest that the ionic radius of Ca^{2+} may play an important role in membrane fusion. However, there is a major spatial problem. As discussed earlier, calcium ions [Ca^{2+}] exist in their hydrated state within cells. Hydrated calcium [$\text{Ca}(\text{H}_2\text{O})_n]^{2+}$ has more than one shell around the Ca^{2+} , with the first hydration shell around the Ca^{2+} being the strongest and having six water molecules arranged in an octahedral configuration [2] and measuring $\sim 6 \text{ \AA}$ (Fig. 2). Studies further reveal that in the hydrated Ca^{2+} ion, depending on its coordination number, the nearest average neighbour Ca^{2+} -O and Ca^{2+} -H distances at $r \sim 2.54 \text{ \AA}$ and $r \sim 3.2 \text{ \AA}$ respectively in the first hydration shell. How then would it be possible for a hydrated calcium ion, measuring $\sim 6 \text{ \AA}$, to fit between the 2.8–3 \AA space between apposing bilayers established by the t-/v-SNARE complex? One possibility would be that calcium has to be present in the buffer solution when t-SNARE vesicles and v-SNARE vesicles meet. In that case, if t- and v-SNARE vesicles are allowed to mix in a calcium-free buffer, no

fusion should occur when calcium is added. This was tested in the current study.

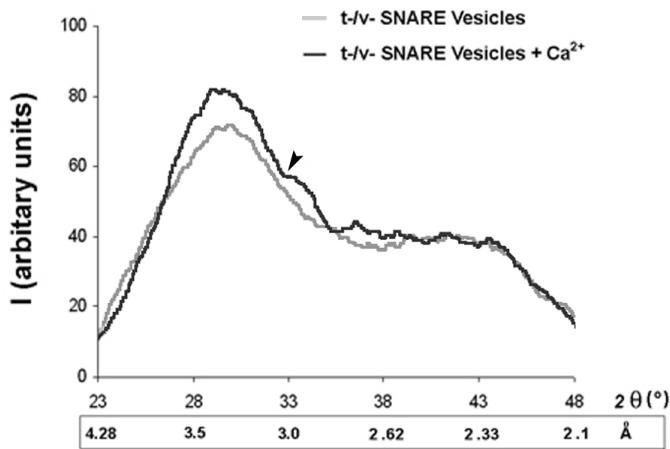


Figure 1. Wide-angle X-ray diffraction patterns of interacting SNARE vesicles. Representative diffraction profiles from one of four separate experiments using t- and v-SNARE-reconstituted lipid vesicles, both in the presence or absence of 5 mM Ca^{2+} is shown. The arrow marks the appearance of a new peaks in the X-ray diffractogram, following addition of calcium.

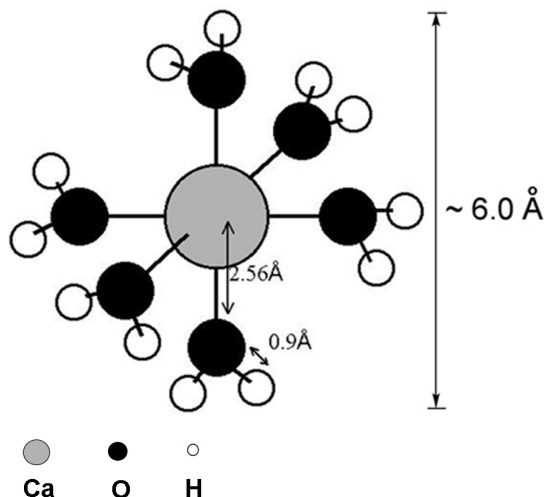


Figure 2. Schematic diagram of a hydrated calcium ion with only the first hydration shell shown. The calcium ion with its six water molecule ligands in an octahedral arrangement, and the bond distances, are shown.

Light scattering experiments (Fig. 3) were performed on t-SNARE- and v-SNARE-reconstituted phospholipid vesicles, in the presence and absence of calcium, and in the presence of NSF and ATP. NSF (N-ethylmaleimide-sensitive factor) is an ATPase that is known to disassemble the t-/v-SNARE complex. As outlined in the methods section, using the light scattering measurements aggregation and membrane fusion of lipid vesicles were monitored on the seconds timescale [5, 7]. The initial rapid increase in intensity of light scattering was initiated by the addition of t- and v-SNARE vesicles

into a calcium-free buffer solution. A slow decay of light scattering (Fig. 3), representing interactions between t-/v-SNARE vesicles, was observed. Addition of calcium at a later time (arrowhead in Fig. 3) has little effect on light scattering. These studies show that if t-SNARE vesicles and v-SNARE vesicles are allowed to interact prior to calcium addition, no significant change in light scattering is observed since there is little or no fusion between the vesicles. On the contrary, when calcium is present in the buffer solution prior to addition of the t-SNARE and v-SNARE vesicles, there is a marked drop in light scattering, as a result of rapid vesicle aggregation and fusion (Fig. 3). However, in the presence of NSF-ATP in the assay buffer containing calcium, a significant inhibition of aggregation and fusion of proteoliposomes was observed (Fig. 3) due to t-/v-SNARE disassembly by NSF. NSF, in the absence of ATP, had no effect on the light scattering properties of the vesicle mixture (data not shown). These results suggest that NSF-ATP disassembles the SNARE complex, thereby reducing the number of interacting vesicles in solution. The disassembled trans-SNARE complex leave the apposed vesicles widely separated, out of reach for establishing the Ca^{2+} -phosphate bridges required for membrane fusion (Fig. 3). Similarly, if the restricted area between adjacent bilayers formed by the circular arrangement of the t-/v-SNARE complex [6] is preformed, then hydrated Ca^{2+} ions would be too large (Fig. 2) to be accommodated between bilayers and hence subsequent addition of Ca^{2+} would have little or no effect (Fig. 3). However, when t-SNARE vesicles interact with v-SNARE vesicles in the presence of Ca^{2+} , the t-/v-SNARE complex formed allows formation of calcium-phosphate bridges between opposing bilayers, leading to the expulsion of water shells around the Ca^{2+} ion, leading to local dehydration, membrane destabilization, lipid mixing and membrane fusion (Fig. 3). These results have further been confirmed by electrophysiological measurements in experiments using reconstituted bilayer and proteoliposome fusion assays (data not shown).

In summary, our X-ray and light scattering studies demonstrate that calcium bridging of the apposing bilayers is required to enable membrane fusion. This calcium bridging of apposing bilayers leads to the release or expulsion of water from the hydrated Ca^{2+} ion, in turn leading to bilayer destabilization and membrane fusion. It could also be argued that the binding of calcium to the phosphate head groups of the apposing bilayers would form a stronger bond than the Ca^{2+} -water complex, resulting in the displacement of water from the water shell around the Ca^{2+} , as well as from the loosely coordinated water around the phospholipid head groups,

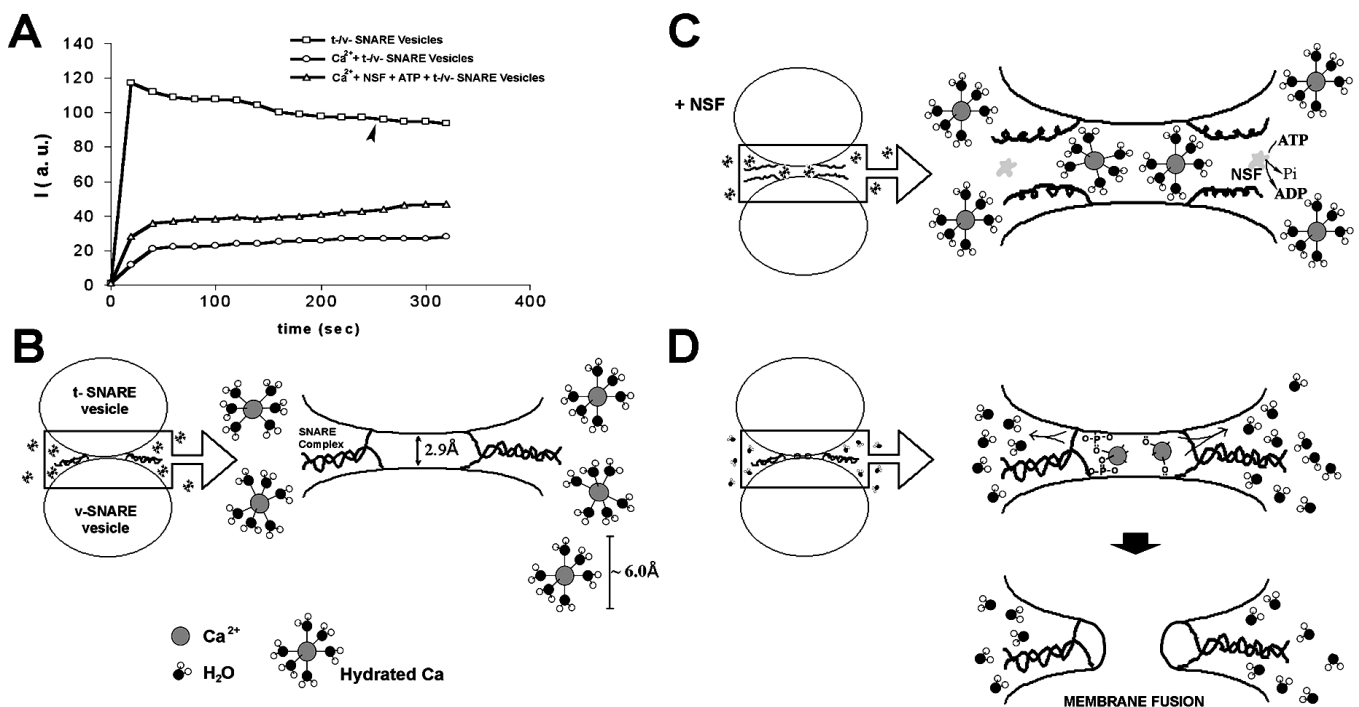


Figure 3. Light scattering profiles of SNARE-associated vesicle interactions. **A,B.** Addition of t-SNARE and v-SNARE vesicles in calcium-free buffer lead to a significant increase in light scattering. Subsequent addition of 5 mM Ca²⁺ (marked by the arrowhead) does not have any significant effect on light scattering (\square). **A,C.** In the presence of NSF-ATP (1 μ g/ml) in the assay buffer containing 5 mM Ca²⁺, significantly inhibited vesicle aggregation and fusion (\triangle). Pi denotes inorganic phosphate. **A,D.** When the assay buffer was supplemented with 5 mM Ca²⁺, prior to the addition of t- and v-SNARE vesicles, it led to a 4-fold decrease in light scattering intensity due to Ca²⁺-induced aggregation and fusion of t-/v-SNARE-apposed vesicles (\circ). Light scattering profiles shown are representatives of 4 separate experiments.

leading to destabilization of the lipid bilayer. Additionally, the energy gained in Ca²⁺-phosphate interaction is large enough to overcome the repulsive interbilayer forces, further contributing to membrane fusion.

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