THE EXOTHERMIC REACTION OF CALCIUM WITH UNILAMELLAR PHOSPHATIDYLSERINE VESICLES

Titration microcalorimetry

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1. Introduction

Phospholipid membranes have been used extensively as model systems for understanding the mechanism of membrane fusion in biological systems (reviews [1,2]). Phosphatidylserine (PS) membranes have unusual properties with respect to their interaction with divalent ions. For example, planar bilayer membranes (black lipid films) are unstable if Ca^{2+} is added to only one side of the membrane [3,4]. Small unilamellar PS vesicles have a similar instability in the presence of Ca^{2+} in that they fuse [5], leak their contents [3], and eventually form cochlear cylinders [6]. PS also exhibits a specificity for the binding of Ca^{2+}, and the gel to liquid crystalline transition temperature of the phospholipid is shifted to above 100°C when Ca^{2+} is added [7,8]. The binding of divalent ions to PS appears to be correlated with their ability to fuse sonicated vesicles made of this phospholipid [8–11]. The addition of Ca^{2+} to these vesicles results in the release of heat as measured in a batch calorimeter [8]. To identify the source of the heat released and its possible involvement in the fusion reaction requires a detailed knowledge of the relationship of the extent of the exothermic reaction and the amount of Ca^{2+} bound to the vesicles. For this purpose, we have measured the heat of reaction in a titrating microcalorimeter and have determined the Ca^{2+} bound by means of a Ca^{2+}-specific electrode. The thermometric titration method allows the determination of the energy liberated as a function of the extent of the reaction in a single experiment and is equivalent to a large number of experiments with conventional batch calorimetry [12,13]. In the latter, many reactions are occurring simultaneously upon introduction of a certain Ca^{2+} level, whereas in titration calorimetry the heat associated with the addition of small increments of Ca^{2+} can be determined.

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2. Materials and methods

Phosphatidylserine was isolated from bovine brain [14] and shown to be pure by two-dimensional thin-layer chromatography. Unilamellar vesicles were prepared by sonication. The lipid was dried in vacuum, hydrated with 100 mM NaCl, 2 mM L-histidine, 2 mM *N*-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) (pH 7.4) vortexed for 5 min and sonicated under Argon in a bath-type sonicator maintained at 20°C. The clear suspension was centrifuged for 1 h at 114 000 × *g* to remove any multilamellar or large vesicles and the upper 4/5 of the supernatant was used for the experiments. The lipid concentration was determined by phosphate analysis [15].

Thermometric titrations were performed in a Tronac constant temperature environment titration calorimeter at 25°C as in [12]. The reaction chamber contained 2.5 ml of 2 mM phospholipid, and Ca^{2+} was titrated at a constant rate of 18 μl/min from a stock solution of 100 mM Ca^{2+} in the same buffer as the vesicle suspension.

Ca^{2+}-binding to the vesicles was determined by measuring the free Ca^{2+} with a Ca^{2+} specific electrode (Orion Research, Model SS-20). Different amounts of the divalent cation were slowly added with a microsyringe to the vesicle suspension (2 mM phospholipid) with rapid mixing to avoid high local concentrations and the mixture allowed to equilibrate at 25°C.

3. Results and discussion

A representative microcalorimetric titration of unilamellar PS vesicles with Ca^{2+} is shown in fig.1. The addition of up to 0.25 Ca^{2+}/PS produced a slight endothermic reaction. Ca^{2+}-binding experiments showed that ~0.15 Ca^{2+} was bound/PS at this point in the titration. If the titration was stopped here no further change in the heat was observed. When the Ca^{2+} concentration was increased an exothermic component appeared, and as the added Ca^{2+}/PS reached 0.7 the Ca^{2+} dependence of the exothermic reaction increased drastically. The reaction was complete at ~1.5 Ca^{2+} added/PS at which point the total heat released was 4.7 ± 0.5 kcal/mol phospholipid. It should be noted, however, that since the titration and the reaction are proceeding simultaneously, the amount of titrant may exceed the amount actually necessary to bring about the sharp increase in the released heat. Indeed, if the titration is terminated at an early stage of the exothermic reaction (at ~0.4 Ca^{2+} added/PS), the reaction continues beyond this point, although not to completion. Therefore, the reaction can be initiated by lower amounts of Ca^{2+} added/PS than the value at the point of the steepest increase in heat release. We are currently investigating this phenomenon in detail. In parallel experiments a discontinuous increase in Ca^{2+} binding occurred above this titration point: The Ca bound/PS increased from 0.22 at 0.4 Ca^{2+} added/PS to 0.39 when the Ca^{2+} added/PS was raised to 0.6. This change in the Ca^{2+} binding properties of PS vesicles is probably associated with:

(i) The aggregation of the vesicles and the formation of a Ca^{2+}/PS complex with a higher binding constant [8] compared to Ca^{2+} binding to non-aggregated vesicles;

(ii) The availability of the PS molecules in the inner monolayer for complexation with the divalent ion, since aggregation and fusion result in an increased permeability of the membrane and the release of internal aqueous contents [5,8,16], allowing Ca^{2+} to enter the vesicle interior during this process.

These observations are in agreement with the results obtained with batch calorimetry, in that an exothermic reaction is observed above a threshold concentration of Ca^{2+}. The enthalpy of the reaction is slightly lower than that reported in [8] (5.5 ± 0.5 kcal/mol). Titrating microcalorimetry, however, gives the detailed dependence of this reaction on the Ca^{2+} added to the PS vesicles, demonstrating the usefulness and efficiency of this method for investigating divalent cation interactions with phospholipid membranes.

The initial stages of heat release appears to be associated with the first contact and fusion of the PS vesicles. It has been postulated that the drastic phase change induced by Ca^{2+} in PS bilayers is dependent on the establishment of a Ca^{2+} bridge between 2 bilayers [8]. At the early stages of fusion only a limited fraction of the PS bilayer would be involved in an inter-bilayer contact. Initial fusion events are accompanied by the release of ~10% of the internal contents of the vesicles [16] with the concomitant exposure of the inner monolayer to Ca^{2+}. As fusion proceeds the vesicle structure collapses and all the phospholipid becomes available for complexation with Ca^{2+}. We believe it is this latter process which gives rise to the major increase in heat release above 0.7 Ca^{2+} added/PS. At this stage, Ca^{2+} has formed an anhydrous complex with the PS, accompanied by a transformation of the phospholipid acyl chains (mostly 18:0 and 18:1 [14]) from a hexagonal packing to a crystalline orthorhombic perpendicular packing [7,8,17–19]. The

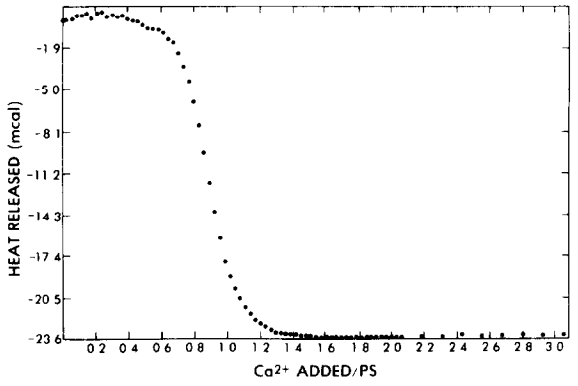

Fig.1. Thermometric titration of PS vesicles with Ca^{2+}. Small (~250 Å diameter) unilamellar PS vesicles were suspended in 2.5 ml 100 mM NaCl, 2 mM L-histidine, 2 mM TES, pH 7.4, at a lipid concentration of 2 mM. T = 25°C. Negative values of the enthalpy indicate an exothermic reaction.

total heat released during the titration, 4.7 ± 0.5 kcal/mol, is similar to the enthalpy of the liquid-crystalline–gel transition of multilamellar dispersions of PS in NaCl (4.5 ± 0.5 kcal/mol) determined by differential scanning calorimetry [7,19] and may reflect the crystallization of the acyl chains. It is also likely, however, that part of this heat is due to the release of the strain in the highly curved membrane during fusion.

Experiments on the kinetics of heat release, the interaction of other divalent ions with PS vesicles and the effect of vesicle size are in progress.

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