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Modulation of lysozyme charge influences interaction with phospholipid vesicles

Olaf Zschörnig *, Gerrit Paasche, Cathrin Thieme, Nikola Korb, Klaus Arnold

University of Leipzig, Institute for Medical Physics and Biophysics, Härtelstrasse 16-18, D-04107 Leipzig, Germany

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Abstract

Lysozyme is a globular protein which is known to bind to negatively charged phospholipid vesicles. In order to study the relationship between charge state of the protein and its interaction with negatively charged phospholipid membranes chemical modifications of the proteins were carried out. Succinylation and carbodiimide modification was used to shift the isoelectric point of lysozyme to lower and higher pH values, respectively. The binding of the modified lysozyme to phospholipid vesicles prepared from phosphatidic acid (PA) was determined using microelectrophoresis and ultracentrifugation. At acidic pH of the solution all lysozyme species reduced the surface charges of PA vesicles. Succinylated lysozyme (succ lysozyme) reduced the electrophoretic mobility (EPM) to nearly zero, whereas native lysozyme and carboxylated lysozyme (carbo lysozyme) changed the surface charge to positive values. At neutral pH, the reduction of surface charges was less for carbo lysozyme and unmodified lysozyme. Succ lysozyme did not change the EPM. Unmodified and carbo lysozyme decreased the magnitude of EPM, but the whole complex was still negatively charged. The bound fraction of all modified lysozyme to PA vesicles at high lysozyme/PA ratios was nearly constant at acidic pH. At low lysozyme/PA ratios the extent of bound lysozyme is changed in the order carbo > unmodified > succ lysozyme. Increasing the pH, the extent of bound lysozyme to PA large unilamellar vesicles (LUV) is reduced, at pH 9.0 only 35% of carbo lysozyme, 23% of unmodified lysozyme is bound, whereas succ lysozyme does not bind at pH 7.4 and 9.0. At low pH, addition of all lysozyme species resulted in a massive aggregation of PA liposomes, at neutral pH aggregation occurs at much higher lysozyme/PA ratios. Lysozyme binding to PA vesicles is accompanied by the penetration of lysozyme into the phospholipid membrane as measured by monolayer techniques. The penetration of lysozyme into the monolayer was modulated by pH and ionic strengths. The interaction of lysozyme with negatively charged vesicles leads to a decrease of the phospholipid vesicle surface hydration as measured by the shift of the maximum of the fluorescence signal of a headgroup labeled phospholipid. The binding of bis-ANS as an additional indicator for the change of surface hydrophobicity is increased at low pH after addition of lysozyme to the vesicles. More hydrophobic patches of the lysozyme–PA complex are exposed at low pH. At low pH the binding process of lysozyme to PA vesicles is followed by an extensive intermixing of phospholipids between the aggregated vesicles, accompanied by a massive leakage of the vesicle aqueous content. The extent of lysozyme interaction with PA LUV at neutral and acidic pH is in the order carbo lysozyme > lysozyme > succ lysozyme.

Keywords: Lysozyme; Liposomes; Aggregation; Fusion; Fluorescence spectroscopy; Electrophoretic mobility

Abbreviations: PA, phosphatidic acid; PL, phospholipid; DPE, *N*-(5-dimethylaminonaphtalene-1-sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; Pyr-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphatidylcholine; ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; DPX, *p*-xylene-bis-pyridinium bromide; MLV, multilamellar vesicles; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; mol. wt., molecular weight; EPM, electrophoretic mobility; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; AMPSO, 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxy-propanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid

* Corresponding author. Tel.: +49 341 9715706; fax: +49 341 9715709.
E-mail address: zsco@medizin.uni-leipzig.de (O. Zschörnig).

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1. Introduction

Protein-induced fusion processes are widespread occurring mechanisms in cell biology. In the last decade, the role of the proteins involved in exocytosis like SNARE, SNAP or synaptotagmine was investigated [1]. However, the knowledge about the exact interaction between the opposing outer monolayers of the plasma membranes and the structure, organization and conformation of the fusion proteins is still in the focus of research [2,3]. On the other hand, knowledge about the interaction of proteins with artificial membranes has a large impact on biotechnological applications. Lysozyme is a relatively small protein with a MW of 14.6 kDa and was subjected to several investigations in conjunction with membrane interaction [4–6]. Lysozyme induces fusion of acidic PL vesicles under definite conditions, e.g. fusion can be triggered at low pH and low ionic strength. Arvinte et al. [7,8] coupled lysozyme to uncharged PL vesicles and measured fusion with erythrocytes at low pH. From these measurements the authors concluded that the maximum of lysozyme activity is in the range of about pH 5.0.

In a recent paper, Arnold et al. [4] reported that the lysozyme-induced fusion of phosphatidylserine vesicles is pH dependent and only occurs below pH 5.0. Additionally, the authors observed a penetration of lysozyme into the PL membrane using energy transfer measurements and monolayer techniques. In contrast, Posse et al. [9] reported a fusion efficiency for lysozyme over a broad pH range (pH 4.0–9.0). These authors did not find an internal content mixing of the vesicles, but a massive PL mixing accompanied by vesicle leakage.

In the present paper, we will focus on the mechanism of lysozyme PL interaction especially under the view of the balance between electrostatic and hydrophobic contributions during the aggregation and fusion process of the negatively charged PL membranes.

It has been shown that lysozyme binds strongly to negatively charged membrane surfaces via electrostatic interactions. It has been established that such binding may have both a structural and a functional role, mediated by effects on the conformation of the protein as well as on the structure of the membrane surface [10].

Spectroscopic studies have shown that the binding of lysozyme to phospholipid membranes induces extensive alterations in the conformation of the protein backbone including loosening and destabilizing of the overall protein structure. It was also demonstrated that lysozyme binding alters the structure of the lipid phase of negatively charged membranes [11]. Although the electrostatic interactions between negatively charged membranes and lysozyme are well documented and have been extensively discussed within the context of specific mechanisms of lysozyme binding, there are results that indicate the existence of hydrophobic interactions of the protein with the lipid bilayer. Especially, strong experimental support for this has been provided by the finding that all bound lysozyme cannot dissociate from such membranes, either by increasing the ionic strength of the buffer solution, or by diluting the bulk protein [12]. This behaviour occurs despite the fact that the initial binding itself is very sensitive to both the ionic strength of the solution and the amount of charge of the lipid membrane, consistent with the electrostatic nature of the interaction.

In summary, there appears to be a general consensus on two points:

1. The initial binding step of lysozyme to the surface of a lipid membrane is governed by electrostatic effects.
2. Subsequent to the initial stage of binding, changes in the structure of the protein and lipid components of the membrane occur, leading to a new and more complex behaviour involving penetration of lysozyme into the lipid phase.

2. Materials and methods

Phosphatidic acid (PA) (Avanti Polar Lipids, USA) derived from egg lecithin was used without further purification. The PA batches used were pure as shown by thin layer chromatography. 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS), 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium bromide) (DPX) and fluorophore-labelled phospholipid 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphatidylcholine (Pyr-PC) were obtained from Molecular Probes. *N*-(5-Dimethylaminonaphtalene-1-sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (DPE) was obtained from Sigma. Egg white lysozyme was from Boehringer Mannheim. Succinic lysozyme was prepared according to the procedure as described in [13]. Carboxylated lysozyme was prepared according to [14]. The isoelectric points of the modified lysozyme preparations were determined according to [15], shown in Fig. 1. An isoelectric point of about 4.5 was determined for succ lysozyme using this method, for unmodified lysozyme the isoelectric point occurs at p$I \sim 10.0$ and for carbo lysozyme the pI was at 11. As a second way to measure the modification of lysozyme, we used electrospray ionization fourier transform ion-cyclotron resonance mass spectrometry (FTICR–MS). The measurements were performed on an Apex II FTICR mass spectrometer with 7 T superconducting magnet (Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionization source (Agilent Technologies, Waldbronn, Germany). A Cole-Parmer syringe pump (Cole-Parmer, Niles, IL, USA) was used to infuse the solution at flow rates of 2 μl/min.

Multilamellar vesicles (MLV) were prepared according to Bangham et al. [16]. Small unilamellar vesicles (SUV) were prepared by hydrating the dried phospholipids in the appropriate buffer, vortexing for 10 min and then sonicating for 10 min using a Branson W-250 tip sonifier keeping the temperature of the sample constant at 30 °C. Large unilamellar vesicles (LUV) were produced by extrusion