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Quantitative fluorescence analysis of the adsorption of lysozyme to phospholipid vesicles

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Abstract. Experiments directed to measure the interaction of lysozyme with liposomes consisting of phosphatidylcholine (PC) and phosphatidylserine (PS) have been conducted by monitoring both protein and lipid fluorescence and fluorescence anisotropy of the protein. The binding of lysozyme to the unilamellar vesicles was quantified using a novel method of analysis in which the fractional contribution at moderate binding conditions is determined from either total fluorescence decay or anisotropy decay curves of tryptophan at limiting binding conditions. In the energy transfer experiments PC and PS lipids labelled with two pyrene acyl chains served as energy acceptors of the excited tryptophan residues in lysozyme. The binding was strongly dependent on the molar fraction of negatively charged PS in neutral PC membranes and on the ionic strength. Changes in the tryptophan fluorescence decay characteristics were found to be connected with long correlation times, indicating conformational rearrangements induced by binding of the protein to these lipid membranes. The dynamics of membrane bound protein appeared to be dependent on the physical state of the membrane. Independent of protein fluorescence studies, formation of a protein-membrane complex can also be observed from the lipid properties of the system. The interaction of lysozyme with di-pyrenyl-labelled phosphatidylserine in anionic PS/PC membranes resulted in a substantial decrease of the intramolecular excimer formation, while the excimer formation of dipyrenyl-labelled phosphatidylcholine in neutral PC membranes barely changed in the presence of lysozyme.

Key words: Protein-lipid interaction – Lysozyme – Conformation – Dipyrenyl phospholipids – Fluorescence distribution analysis – Fractional analysis – Membrane perturbation – Resonance energy transfer

Introduction

The interaction of proteins with lipids is relevant for a wide range of biological and biotechnological processes. For example, various peripheral proteins are activated upon binding to membranes. To elucidate the principles that govern protein adsorption to lipid interfaces and conformational stability upon absorption, it is necessary to examine simple, well-defined systems and to have methods available for quantification of the absorbed amounts. Since lysozyme is a stable, well-characterised protein with known 3-dimensional structure having six tryptophanyl residues (Imoto et al. 1972), it can easily serve as a model protein in these interaction studies. In this paper we report on the rotational dynamics of lysozyme molecules interacting with phospholipid vesicles derived from the decays of tryptophan fluorescence anisotropy. The dependence of this interaction on the ionic strength and on the membrane surface charge was investigated. Reversible association of lysozyme to the membrane was also studied by monitoring resonance energy transfer from excited tryptophans in lysozyme to pyrene labelled phosphatidylserine (PS) and phosphatidyl-choline (PC). Both techniques have proven to be very sensitive for following protein association at membrane surfaces (Pap et al. 1993; Bastiaens et al. 1993; Omata and Friedman 1991; Vincent and Gallay 1991; Houbre et al. 1990). However, precise quantitation of the adsorbed amount of protein is often a problem in such studies. In fluorescence lifetime distribution analysis, no unique lifetime class of tryptophan residues arises from interaction of lysozyme with membrane vesicles, which makes it not very suitable for direct quantitation of protein adsorbed to lipid vesicles. In principle, the average tryptophan fluorescence lifetime can be obtained from the

Abbreviations: dipyr$_4$ *sn-1,2*-(pyrenylbutyl), dipyr$_{10}$ *sn-1,2*-(pyrenyldecanoyl), DMPC, dimyristoyl-phosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PS phosphatidylserine

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fluorescence decay and related to the efficiency of energy transfer to lipid acceptors (Dale et al. 1979). This parameter can then be used to quantify the interaction between protein and quenching lipids (Pap et al. 1993; Bastiaens et al. 1993). A disadvantage of this approach is that the time-resolved information is averaged out. An alternative method is global analysis, in which decay parameters characteristic for the bound or free form are globally linked in a set of experiments (Beechem et al. 1991). This has proved to be a powerful method of analysis, but an interpretation of the fluorescence and anisotropy decays using a specific model is needed, and only a limited number of relaxation parameters can be analysed. The complexity of a system containing multiple intrinsic tryptophan residues does not allow for a detailed photophysical interpretation of the decay. In this paper a simple method is proposed to obtain the fraction of bound protein from time-resolved fluorescence and anisotropy decays provided that the decay curves corresponding to free protein and to a sample where the majority of the protein molecules is bound, are known. Next to this quantitative analysis of lysozyme-lipid interaction, a distribution analysis of the time-resolved tryptophan fluorescence and fluorescence anisotropy indicated changes in the conformation and dynamics of lysozyme accompanying its binding to membranes.

Experimental procedures

Materials

Hen egg white lysozyme was purchased from Boehringer (Mannheim). Bovine brain phosphatidylserine (PS), 1,2-dipalmitoyl-L-phosphatidylserine, 1,2-dimyristoyl-L-phosphatidylcholine and 1,2-dipalmitoyl-L-phosphatidylcholine were obtained from Sigma (St. Louis, MO), Di-('-pyrenedecanoyl)-PC ($dipyr_{10}PC$) and di-('-pyrenebutyryl)-PC ($dipyr_4PC$) were synthesised by methods described previously (Patel et al. 1979). Transphosphatidylation of labelled phosphatidylcholine by phospholipase D yielded di-pyrene PS (Comfurius et al. 1990). Purification of the pyrene labelled lipids was performed with high performance liquid chromatography on a silicic acid column (240×10 mm, LiChroprep Si 60, Merck, Germany). Elution was performed with an increasing methanol gradient (0–40%) in chloroform. All buffers were made with nanopure water. All other chemicals were of analytical grade. Unless otherwise noted, experiments were performed using 20 mM Tris HCl buffer, pH 7.5, 20 μM EGTA at 20 °C.

Vesicle preparation

Small unilamellar vesicles were prepared by sonication using the method of Barenholz et al. (1977). The total phospholipid content was determined by phosphate analysis according to the method of Rouser et al. (1970). The pyrene concentration was determined by measuring the optical density at 342 nm in ethanol/DMSO (75:25 v/v) (ε=39700 M^{-1} cm^{-1}).

Fluorescence methods

Pyrene monomer and excimer fluorescence intensities were recorded on a DMX-1000 spectrofluorometer (SLM Aminco, Urbana, IL). The measurements were corrected for the background emission and the spectral instrument variations. Both excitation and emission monochromator bandwidths were set at 4 nm. Monomer and excimer emissions were detected at 377 nm and 480 nm, respectively. In the excimeric experiments the excitation wavelength was 347 nm, while in the energy transfer experiments dual wavelength excitation was used (290 and 335 nm). The latter experiments were carried out as previously detailed (Pap et al. 1995).

Time-resolved fluorescence measurements were carried out with a time-correlated single photon counting setup as described elsewhere (Pap et al. 1993). The excitation wavelength was 295 nm. Fluorescence was selected using a 3 mm WG 335 cut-off filter (Schott, Mainz), an interference filter at 348.8 nm (Schott, bandwidth 4.8 nm FWHM) and a sheet type polariser (Polaroid type HNP'B). All measurements consisted of a number of sequences of registration of 10 s parallel and 10 s perpendicular polarised emission. After each sample the background of samples in the absence of lysozyme was measured, at one fifth of the time of sample acquisition. p-Terphenyl (Eastman Kodak Co.) dissolved in ethanol served as reference compound (fluorescence lifetime = 1.06 ns (Vos et al. 1987)) to yield the dynamic instrumental response function of the set-up (van Hoek et al. 1985). The sample temperature was 20 °C. The data were collected in a multichannel analyser (Nuclear Data model ND66); 1024 channels were used per experimental decay with a time spacing of 30 ps per channel. After transfer the data were analysed on a Silicon Graphics Personal Iris computer model 4D-35 using the Maximum Entropy Method (MEM, Maximum Entropy Data Consultants Ltd., Cambridge, UK) of analysis yielding fluorescence-lifetime and correlation time distributions of the enzyme (Livesey and Brochon 1987; Brochon et al. 1993; Vincent and Gallay 1991). Two-dimensional methods were used which investigate cross-correlations between the fluorescence lifetime components (τ) and the correlation times (ϕ) (Brochon et al. 1993).

Computational methods

Fractional analysis of decay curves

The goal of analysis using the fractional approach is simply to obtain an accurate value of the fraction of limiting states in a multicomponent system. These limiting states are, for instance, membrane bound and free protein. We assume that the multicomponent system only consists of a mixture of both limiting states. In the remainder of this section we will explore the details of this approach.

The case of energy transfer from tryptophan residues to lipid acceptors

The energy transfer fluorescence experiments will yield two limiting fluorescence decays: a strongly quenched

one, $F(t)_s$, where the majority of the protein molecules are associated with pyrene containing vesicles and a non-quenched decay $F(t)_0$ of the protein in buffer. Provided that only two forms of the protein exist (bound and free), decays measured under moderate binding conditions, $F(t)_x$, can be described as a combination of both limiting decays $F(t)_0$ and $F(t)_s$. Elimination of differences in the instrumental response (experiments were conducted on different days) was achieved by convolution of the linear combination of the ideal (response to δpulse) fluorescence $f(t)_0$ and $f(t)_s$ with the impulse response profile of the intermediate decay, $P(t)_x$. The actual decays $f(t)_0$ and $f(t)_s$ were obtained from transformation of MEM-obtained lifetime distributions of the corresponding experimental decays from the lifetime domain into the time domain:

$$f(t)_{0,s} = \int_0^\infty a(\tau)\, e^{-t/\tau} d\tau \tag{1}$$

where $a(\tau)$ is the contribution of lifetime τ in the decay. The experimental decay under moderate binding conditions can then be described as a combination of two convolution products:

$$F(t)_x = (1-\beta)\, N_0 P(t)_x * f(t)_0 + \beta\, N_s\, P(t)_x * f(t)_s \tag{2}$$

where β and $(1-\beta)$ represent the fractional contributions of the limiting decays to $F(t)_x$. N_0 and N_s are the normalisation factors which eliminate differences in initial total fluorescence intensity in the various experiments ($f_{n(t=0)}/f_{x(t=0)}$).

The case of fluorescence anisotropy in which the protein bound to the membrane is more immobilised than free in solution

Similarly, in time-resolved fluorescence anisotropy titration experiments the characteristic decays at limiting binding conditions can be used to describe the anisotropy decay under moderate binding conditions (R_x). The relationship between the calculated intermediate anisotropy decay and the polarised components ($f_{\parallel n}, f_{\perp n}$) of the limiting decays is given by:

$$r(t)_x = \frac{(1-\beta)\, D(t)_0 + \beta\, D(t)_s}{f(t)_x} \tag{3}$$

where $D(t)_0$ and $D(t)_s$ correspond to $f(t)_{\parallel 0} - f(t)_{\perp 0}$ and $f(t)_{\parallel s} - f(t)_{\perp s}$, respectively. $r(t)_x$ is obtained from the experimental polarised components:

$$F(t)_\parallel = P(t)_x * \left(\frac{2}{3} r(t)_x\, f(t)_x + \frac{1}{3} f(t)_x\right) \tag{4}$$

$$F(t)_\perp = P(t)_x * \left(\frac{-1}{3} r(t)_x\, f(t)_x + \frac{1}{3} f(t)_x\right) \tag{5}$$

Relationship between β and the fraction of bound protein (α)

Until now the fractional contribution was determined by assuming that only two forms are present in samples of various two-component mixtures. To assign the fractional contribution β to the actual fraction of bound protein in the sample (α) the following reasoning must be considered. In a mixed sample, the fluorescence and fluorescence anisotropy properties of free lysozyme will contribute to respectively $F(t)_0$ and $D(t)_0$. Membrane-bound lysozyme molecules contribute to the decays $F(t)_m$ or to $D(t)_m$ which are approached by $F(t)_s$ and $D(t)_s$, but which are, in principle, unknown. Similarly to earlier explanations $F(t)_s$ can be expressed with a linear combination of $(1-x)\, F(t)_0$ and $x\, F(t)_m$. Substituting this expression for $F(t)_s$ into Eqs. (2) and (3) yields a relation between β and the actual fraction of bound protein (α):

$$\beta = \frac{\alpha}{x} \tag{6}$$

If $F(t)_s$ does not correspond to $F(t)_m$ ($x<1$), some type of model-dependent step will need to be included to obtain a value for x which describes the fractional association of the protein to the lipid interface.

Binding model

Free protein (P_f) is considered to be in equilibrium with interaction sites on the membrane. A binding site on the membrane surface is defined as the average number of phospholipid molecules (m) that are occupied by binding of the protein. The fraction of bound protein (α) can then be modelled with a Langmuir adsorption isotherm, which includes the association constant (K_a), the total concentration of protein (P_t), the total concentration of phospholipids (L_t) and m.

$$\nu = \frac{P_b}{0.53\, L_t / m} = \frac{K_a\, P_f}{1 + K_a\, P_f} \tag{7}$$

Here ν is the fraction of occupied sites for the protein at the membrane surface. The factor 0.53 is introduced since only the lipids at the outer leaflet of the bilayer membrane (53% of a 120 nm diameter vesicle) are accessible for lysozyme binding. Since $P_b = \alpha P_t$ and $P_f = (1-\alpha) P_t$ (fractional concentrations) the following expression is obtained for α:

$$\alpha = \frac{m + K_a(0.53L_t + P_t m) - K_a P_t m \sqrt{\frac{-2.12 L_t}{P_t m} + \left(-1 - \frac{1}{K_a P_t} - \frac{0.53 L_t}{m P_t}\right)^2}}{2 K_a m P_t} \tag{8}$$

This function can be applied to the β values obtained with the fractional analysis in order to estimate K_a and x.

Results and discussion

Conformational alterations in lysozyme accompany membrane binding

Although lysozyme contains six tryptophanyl residues, it has been estimated that more than 80% of its fluorescence comes from two residues (Trp-62 and/or Trp-108) (Imoto et al. 1971). Figure 1A shows the results of a two-dimensional maximum entropy analysis of tryptophan fluorescence of lysozyme in aqueous buffer solution describing

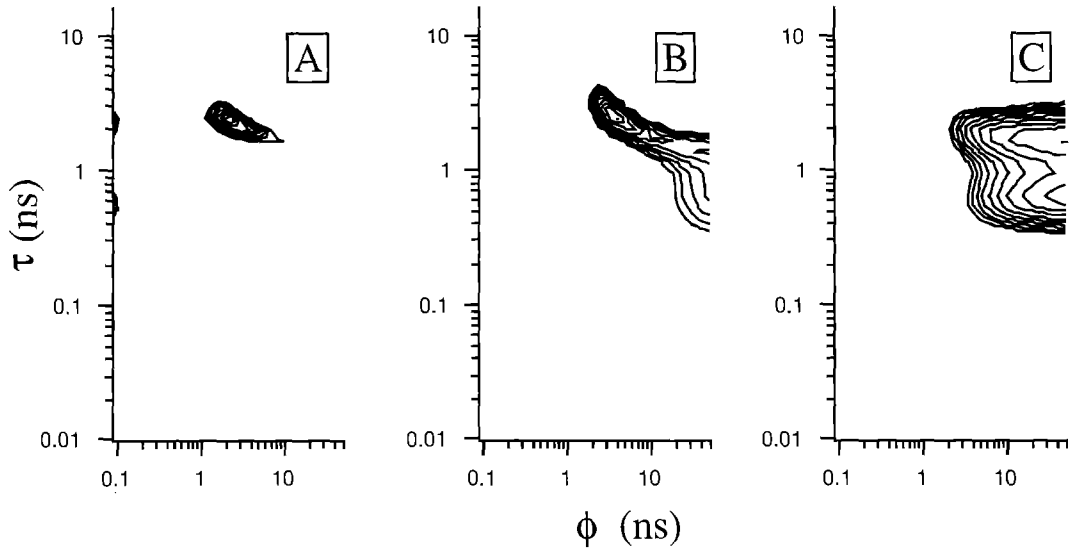

Fig. 1. MEM-recovered contour plots (τ, ϕ) of polarised tryptophan fluorescence of lysozyme in buffer **A**, in the presence of vesicles made from 60 μM DPPC **B** and in presence of 60 μM DPPC/DPPS (3:1) **C**. An initial anisotropy of 0.23, obtained from the one-dimensional anisotropy analysis, was fixed in the two-dimensional analysis

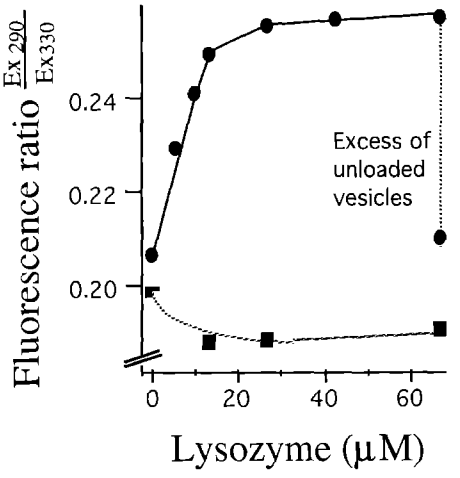

Fig. 2. The dependence of monomer fluorescence ratio at two excitation wavelengths (290 nm/330 nm) of dipyrene lipids on the lysozyme concentration. The vesicles consisted of DOPC/brain-PS/dipyr$_{10}$PS (3:1:1) (●) and DOPC/dipyr$_{10}$PC (4:1) (■). The total lipid concentration was kept constant to 10 μM during the protein titration. After addition of an excess of DOPC/brain-PS without pyrene lipid (final concentration 100 μM) the ratio of monomeric pyrene fluorescence reverses to its initial value, indicating that the binding is reversible

the associative behaviour between fluorescence lifetimes and rotational correlation times. The fluorescence lifetime distribution of tryptophan in lysozyme, is described by two discrete lifetime peaks. A main contour (τ=1.7 ns, ϕ=4.4 ns) in the image accounts for 92% of the emission. The relatively short correlation time (for a protein with molecular mass of 14900 Da) might partly originate from a faster apparent depolarisation due to homo-energy transfer between tryptophan residues within the protein molecule. In the presence of DPPC vesicles, the total tryptophan fluorescence image is quite similar. An additional minor contour (the contour levels are log-scaled) appears at long correlation times indicating a small second population of membrane bound lysozyme molecules with different motional properties (Fig. 1 B). In the presence of anionic membranes consisting of DPPC and DPPS (3:1), however, the contour peak of free enzyme is fully replaced by two other peaks with fluorescence lifetime barycenters of 0.29 and 1.58 ns associated with very long correlation times (Fig. 1 C). Here all lysozyme molecules are immobilised by the lipid membranes. Interactions of lysozyme with vesicles do not only lead to changes in the protein dynamics, but also change the tryptophan fluorescence lifetime distribution (for other examples, see als Vincent and Gallay 1991; Brochon et al. 1993). Since a faster decay of tryptophan fluorescence is characteristic for a "quenched" conformation of the protein we conclude that stabilisation of structural conformers of lysozyme by the membrane leads to the appearance of shorter lifetime components associated with long correlation times. These observations are in agreement with the lipid induced enhancement of helical secondary structure of lysozyme as observed by Lippert and coworkers (Lippert et al. 1980).

Reversibility of binding

In order to investigate reversibility of binding a competition experiment was performed in which pyrene-lipid loaded vesicles interacting with lysozyme molecules were replaced by unloaded vesicles. In this experiment the monomeric pyrene-lipid steady state emission was monitored at 377 nm. The ratio was determined of monomer fluorescence when exciting at the pyrene excitation minimum at 290 nm and at the pyrene excitation maximum at 330 nm. At 290 nm excitation, the pyrene moieties within the Förster radius of lysozyme are mainly excited via resonance energy transfer from primary excited tryptophans. The excitation at 330 nm directly excites pyrene and this signal is used as an internal control to correct for secondary effects like changes in the excimer/monomer intensity ratio. In Fig. 2 the monomer

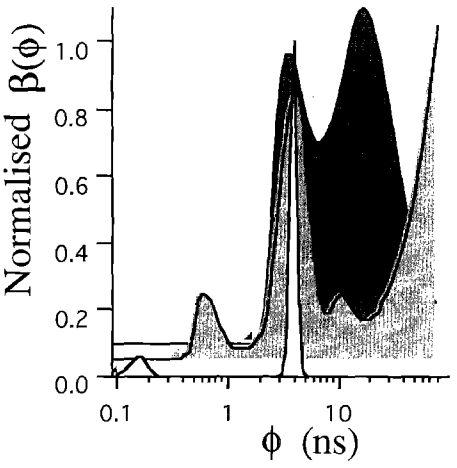

Fig. 3. MEM recovered correlation time spectra from fluorescence anisotropy decay of lysozyme in the presence of DPPC vesicles (□), vesicles of DPPC/DPPS (70:30) ( ) and vesicles of DOPC/brain-PS (70:30) (▒). The used lysozyme and lipid concentrations were 1.5 μM and 60 μM, respectively

fluorescence ratio is plotted for DOPC/brainPS/dipyr$_{10}$PS (3:1:1) and DOPC/dipyr$_{10}$PC (4:1) as a function of the lysozyme concentration. In the case of the anionic membranes, the monomer intensity ratio increases with lysozyme concentration, indicating an increase of sensitised pyrene excitation via lysozyme, and thus illustrating membrane binding of the protein. Saturation of this effect is achieved. When a 10-fold excess of anionic vesicles without pyrene lipids is added, the ratio reduces to almost its original value of 0.21. This indicates that the binding of lysozyme to charged membranes is essentially reversible. In the case of the neutral PC membranes no systematic effect is observed upon addition of lysozyme, indicating the absence of binding to the membrane.

The reversibility of binding is not in accordance with previous studies using solid hydrophobic surfaces (Schmidt et al. 1990; Horsey et al. 991) or charged silica as adsorption surface (Horsey et al. 1991). Both artificial surfaces, however, induce more extended conformational adaptations causing irreversibility of binding (Schmidt et al. 1990; Horsey et al. 1991). In contrast to solid silica surfaces where the charges are strongly localised, the charged lipid headgroups can adapt their position in the membrane with respect to opposite charges at the protein surface.

The effect of the membrane state on bound lysozyme dynamics

The influence of lipid phase of the membrane (gel or fluid) on the fluorescence and dynamic behaviour of lysozyme was investigated by measuring the fluorescence and anisotropy decays in the presence of model membrane systems of DPPC (gel state, neutral), DPPC/DPPS (70:30) (gel state, negatively charged) or DOPC/brain-PS (70:30) (fluid, negatively charged). Correlation time distributions, obtained from MEM analysis of the fluorescence anisotropy decays of lysozyme in the presence of these vesicles having different states of the membrane are presented in Fig. 3.

In the presence of the electrically neutral DPPC vesicles, the anisotropy decay is essentially described by a single correlation time distribution characteristic for enzyme in aqueous solution. In the presence of charged fluid state membranes (DOPC/brain-PS (3:1)) an additional correlation time peak appears at approximately 30 ns, which originates from lysozyme bound to these membranes. The correlation time of lysozyme interacting with membranes in the gel state (DPPC/DPPS (3:1)) is increased to an infinitely long correlation time. Although the accuracy of such long correlation times determined from tryptophan fluorescence is low, the results indicate that the rotation of lysozyme associated to DOPC/brain-PS membranes is essentially isotropic and in the case of DPPC/DPPS vesicles anisotropic. The physical state of the membrane thus significantly affects the dynamic behaviour of peripherically associated lysozyme. This effect can be due to differences in headgroup packing in the different lipid phases or, alternatively, to differences in hydrophobic interaction between lysozyme and lipid acyl chains.

Dependence of lysozyme-membrane association on mixed lipid concentration from quenching of tryptophan fluorescence by resonance energy transfer

In order to evaluate the binding of lysozyme to acidic membranes the polarised fluorescence decays of lysozyme were monitored in the presence of various amounts of vesicles composed of DPPC/DPPS/dipyr$_4$PC (67.5:27.5:5). In the presence of these pyrene loaded vesicles, the tryptophan fluorescence is quenched (Fig. 4 A, lower panel) and reduction of the barycenter value for each lifetime class is observed (data not shown). The titration data were analysed in two steps in order to obtain a value for the binding constant (K_a) of lysozyme for the mixed lipid membranes. In the first step the dependence of the fractional contribution β on the concentration of mixed lipid, was obtained by applying the fractional approach of fluorescence analysis (see Eq. (2)) to all these fluorescence decays. In this analysis the fluorescence decay of lysozyme in buffer represented the non-quenched decay $F(t)_0$, while its decay in a sample with 0.5 mM of mixed lipid was assumed to approximate the decay of lysozyme molecules bound to the vesicle membranes. The lower panel of Fig. 4 A shows both limiting fluorescence decays with their fitted curves and an example of an intermediately quenched fluorescence decay in the presence of 0.17 mM of the mixed lipid. The upper panel shows weighted residuals of the corresponding analyses. The fractional contribution β obtained from the analysis of titration experiments with this approach and corresponding reduced χ^2 values are presented in Fig. 4 B. The average value of the reduced χ^2 obtained from all analyses corresponds to 1.27, which is an acceptable fit quality, especially when we take into account the χ^2 values of 1.13 and 1.21 obtained with the distribution analysis (with MEM) of the limiting decay curves ($F(t)_0$ and $F(t)_s$). Since β is proportional to the fraction of lysozyme bound to the membranes, these experiments clearly

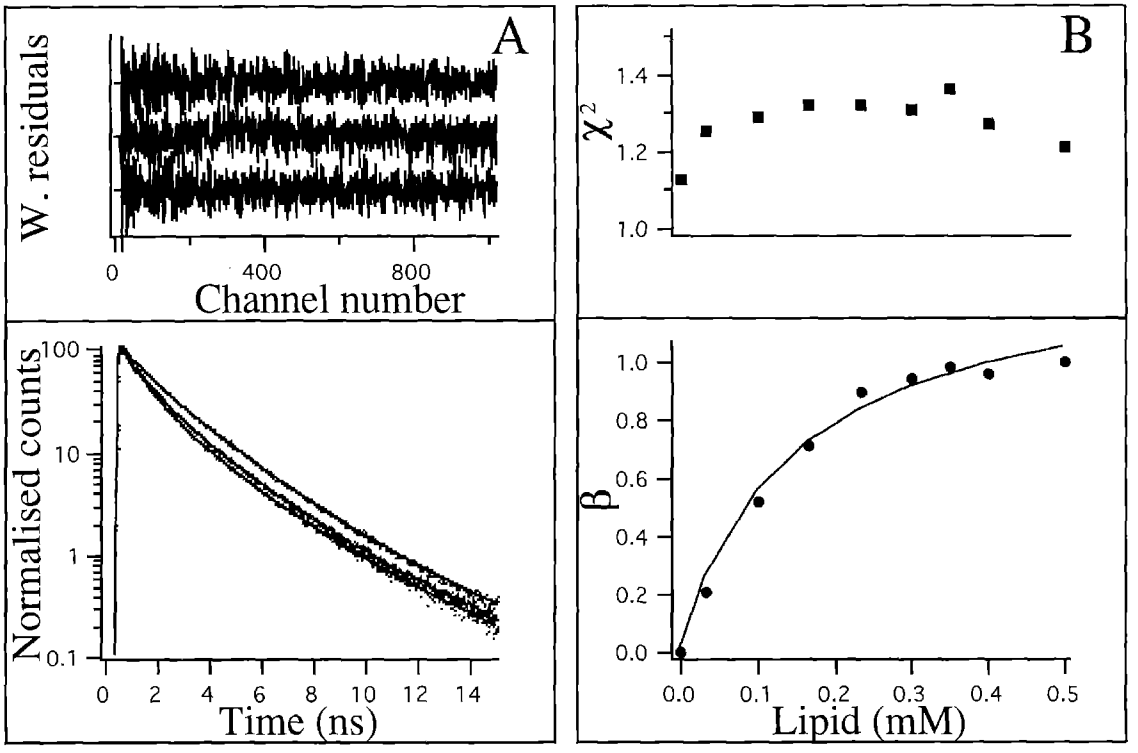

Fig. 4A. The lower panel shows the fluorescence decay profiles (*dots*) of lysozyme in the absence (*non-quenched*) and in the presence (*quenched*) of different amounts of mixed lipid vesicles composed of DPPC/DPPS/dipyr$_4$PC (67.5:27.5:5). The (*solid*) curves are the best fits to the experimental data obtained by fractional analysis. The intermediate decay was analysed with a linear combination of the limiting decays yielding an optimised value for β of 0.71. The weighted residuals of the analyses to the corresponding curves are given in the upper panel. **B** The dependence of the fractional contribution (β) on the mixed lipid concentration is plotted in the lower panel and the fit quality (reduced χ^2) to the experimental fluorescence decay data corresponding to each β value is presented in the upper panel. In this lipid titration experiment, a lysozyme concentration of 1.5 μM was used. A simple one-step binding model was applied to the dependence of β on the mixed lipid concentration (*solid line*). See text for further details

show the gradual increase of bound lysozyme molecules with increasing amounts of mixed lipid present in the sample.

In the second step the dependence of β on the lipid concentration was modelled by applying a simple one-step binding model (described in the computational method section) yielding an optimised value for the fractional contribution x (of $F(t)_s$ in $F(t)_m$) and a value for the binding constant K_a. In this analysis we assumed that $m=14$ (this value is based on a protein radius of 1.7 nm (Blake et al. 1965) and a lipid area of 0.7 nm^2 (Demel et al. 1967)). The analysis yielded a value of 0.83 for x and 2.5×10^5 M^{-1} mixed lipid for K_a. From the preceding results we have seen that membrane binding is accompanied by structural changes in the protein. Therefore the one step binding process assumed in this analysis is a first approximation and the optimised values for x and K_a should be considered as rough estimates.

Dependence of the membrane association of lysozyme on membrane surface charge and ionic strength as determined from fractional analysis of the fluorescence anisotropy

The dependence of lysozyme binding on membrane surface charge was studied by monitoring the polarised tryptophan fluorescence decays at various mole fractions of DPPS in mixed DPPC/DPPS vesicles. The polarised decays were analysed with the fractional approach of anisotropy analysis (see Eq. (3)). The decays of lysozyme in the presence of pure DPPC vesicles and DPPC/DPPS (70:30 molar ratio) vesicles were taken to represent the decay of non-bound lysozyme and of membrane-bound lysozyme, respectively. The lower panel of Fig. 5A shows a typical decay of polarised fluorescence components ($I_{//}$ and $I_{\perp}$) of lysozyme in the presence of DPPC/DPPS vesicles at moderate binding conditions. The solid curve represents the fit obtained with the fractional analysis to the experimental points. The weighted residuals of the corresponding fit are presented in the upper panel. The results of fractional analysis as a function of the mol% PS and the χ^2 values of the fits are presented in Fig. 5B. Increasing the molar fraction of PS in the mixed neutral/anionic membranes causes a gradual enhancement of binding of lysozyme. The enhanced binding might be due to an improved affinity of lysozyme for the membrane with increasing PS mole fraction or to an increase of the number of interaction sites in the membrane.

In order to demonstrate that the specificity of lysozyme for PS is ionic in nature, the polarised fluorescence decays of lysozyme in the presence of 60 μM DPPC/DPPS (70:30) vesicles were determined as a function of ionic strength by adding 0 to 500 mM NaCl to the sample.

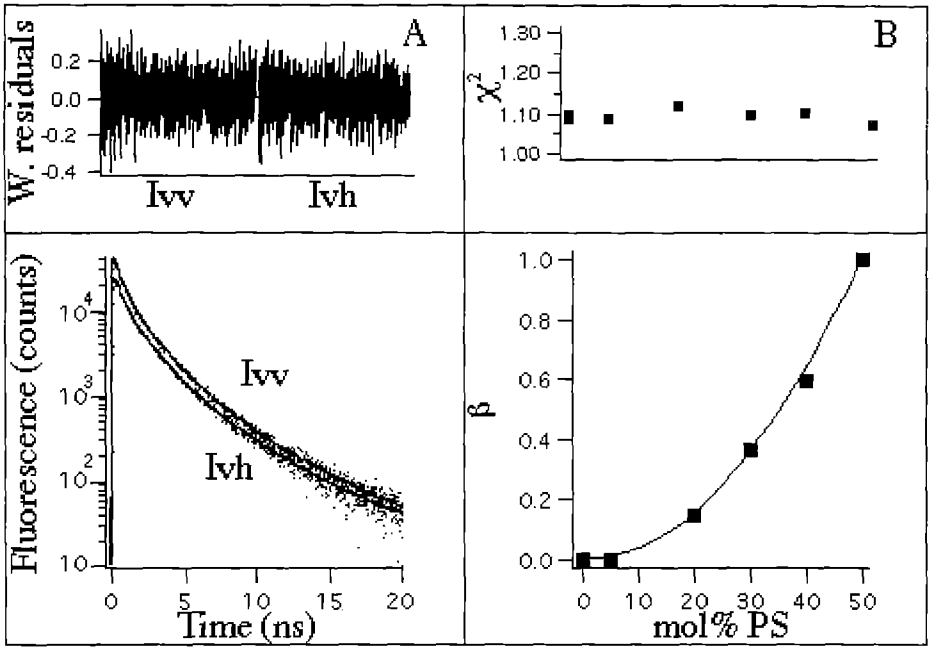

Fig. 5. Dependence of fraction of bound lysozyme on the mole fraction of DPPS in DPPC vesicles. The data points are obtained from fractional analysis of the fluorescence anisotropy data sets. In this experiment the total lipid and lysozyme concentrations were 60 μM and 1.5 μM, respectively

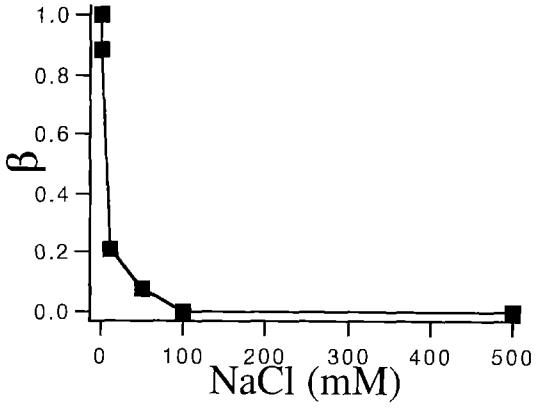

Fig. 6. Effect of ionic strength variation on the fraction of bound lysozyme. Vesicles (60 μM of DPPC/DPPS (70/30)) and lysozyme (1.4 μM) were incubated at various ionic strength (NaCl concentrations from 0 to 500 mM). The data were obtained from fractional analyses of the anisotropy decays of the various samples

Fractional analysis of these anisotropy data yielded β as a function of NaCl concentration (Fig. 6). In this analysis a sample containing protein and membrane together with 500 mM NaCl represented non-associated protein while one without NaCl consistend of mainly associated lysozyme. It is apparent from Fig. 6 that the amount of binding of lysozyme to DPPC/DPPS vesicles decreases with increasing ionic strength of the buffer which indicates that electrostatic interactions play a dominant role in the association of lysozyme with phospholipid bilayers.

Perturbation of the membrane bilayer

To investigate the effect of lysozyme association on the physical properties of the lipid environment, the excimer-monomer fluorescence intensity ratio (E/M) of dipyr$_{10}$PC and dipyr$_{10}$PS was monitored as a function of lysozyme concentration in DOPC and DOPC:brainPS (3:1) vesicles, respectively. Formation of an intramolecular excimer of two pyrene moieties that are attached to the same lipid molecule arises from a collisional encounter between an excited and a non-excited pyrene moiety (Galla and Sackman 1974), and the frequency of such events is governed by the rotational dynamics of the pyrene chains, the motional freedom superimposed by the local lipid environment and the geometry of the parent molecule (Cheng et al. 1991).

Binding of lysozyme leads specifically to a reduction of excimer formation of dipyr$_{10}$PS, and not of dipyr$_{10}$PC, confirming the specificity for acidic lipids in the interaction. The results of this experiment are presented in Fig. 7. Addition of NaCl to a final concentration of 100 mM abolishes the association process and the E/M ratio of dipyr$_{10}$PS recovers almost to its value in the absence of protein. This observation can be interpreted as a decrease in the intramolecular collision frequency of the two pyrene fluorophores in PS. The binding of a protein at the membrane surface may affect acyl chain motion due to condensing or disrupting effects on the head-group packing. These effects may alter the lipid chain motion by modifying the free volume accessible to each lipid molecule. Partial exclusion of DOPC in regions where lysozyme interacts with the membrane might also contribute to changes in the membrane fluidity in these regions and, consequently, influence the intramolecular excimer formation of dipyrene labeled PS.

Conclusions

The major purpose of the present study is to demonstrate that a fractional analysis of fluorescence decay curves can

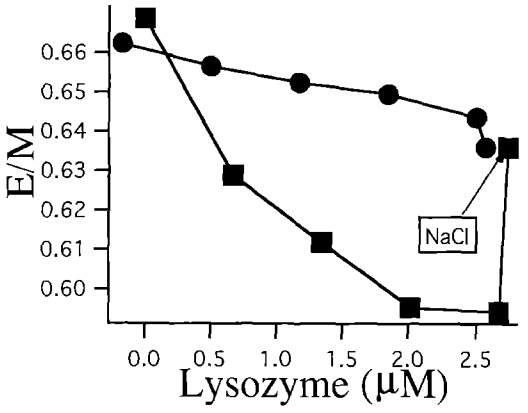

Fig. 7. The dependence of the excimer-monomer fluorescence intensity ratio (E/M) of dipyr$_{10}$PS and dipyr$_{10}$PC on the concentration of lysozyme. The total lipid concentration was kept constant (10 μM). Points ● show the effect on neutral PC/dipyr$_{10}$PC (100:1) vesicles and points ■ that of DOPC/brain-PS/dipyr$_{10}$PS (75:25:1) membranes. In the latter case, reversibility of this acyl chain effect is demonstrated by addition of an excess of NaCl to a final concentration of 100 mM

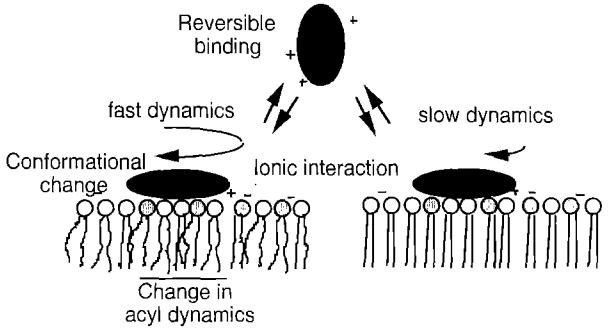

Fig. 8. Schematic representation of the interaction of lysozyme with negatively charged phospholipid vesicles below (*right*) and above (*left*) the phase transition

provide accurate binding curves of proteins to lipid interfaces. The results of the analyses enable the investigation of macromolecular adsorption in a very detailed manner. The fractional analysis has several advantages: 1) A physical interpretation of the observed fluorescence or anisotropy decays in terms of decay parameters is omitted; 2) the maximum amount of information is conserved since the whole decay is used, and not some "average" parameter such as the average fluorescence lifetime; 3) the number of adjustable parameters is minimal; 4) both total fluorescence (sum of polarised decays) and anisotropy (difference of polarised decays) should yield independently identical fractions of the limiting decays. We think that this way of analysis will be applicable not only for the investigation of macromolecular adsorption, but for any multiple component system.

Another result arising from this work is the characterisation of conformational and dynamic aspects of lysozyme adsorption to lipid membranes. The experimental findings are schematically summarised in Fig. 8. Lysozyme exhibits a very weak non-specific binding to bilayers of pure PC and a strong binding to PC containing negatively charged PS. In the latter case the protein can be detached by increasing the ionic strength, indicating that the selectivity for PS is mainly of an electrostatic nature. The rotational dynamics of lysozyme molecules are significantly faster when the protein is adsorbed to fluid membranes rather than to membranes in the gel state. Interaction with the charged membrane surface leads to a change in protein conformation. From the membrane point of view the E/M results indicate that the adsorption of lysozyme changes the structural and/or dynamic properties of the membrane. Effects of peripheral protein binding on lipid motion have been observed by others (See Jain and Zakim 1987; Marsh 1991). In general, the overall result of this surface interaction is an increase of lipid packing density, which reduces the extent of lipid chain motion as recorded by the intramolecular excimer formation.

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