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Electrostatic Interaction between Anions Bound to Site I and the Retinal Schiff Base of Halorhodopsin†

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ABSTRACT: The influence of different anions on the deprotonation of the retinal Schiff base of halorhodopsin in the dark was investigated. We find that a large number of anions cause a significant increase of the pK_a of the Schiff base, an effect attributed to binding to "site I" on the protein. The concentration dependencies of the spectroscopic shifts associated with the changes of the pK_a yielded dissociation constants (and thus binding energies) for the anions, which were related to the Stokes radii. The data fit the predictions of electrostatic interaction between the anions and the positive charge associated with site I, if the latter is located within a few angstroms from the surface of the protein. The specificity of site I toward various anions is quantitatively explained by the differences in the change of Born energy upon transfer of the anions from water to the binding site. The changes in the deprotonation energy of the Schiff base upon the binding of anions, $\Delta\Delta G_{deprot}$, could be calculated from the ΔpK_a at infinite anion concentration. Unexpectedly, the $\Delta\Delta G_{deprot}$ values were remarkably close to the energies of binding to site I. Thus, site I and the Schiff base are strongly electrostatically coupled, either because of close proximity or because of the possibility of allosteric energy transfer between them.

The existence of three different anion binding sites in halorhodopsin, the light-driven chloride ion pump of *Halobacterium halobium*, has been demonstrated previously (Lanyi & Schobert, 1983; Schobert et al., 1983, 1986; Steiner et al., 1984; Falke et al., 1984). One of these sites, which is specific for chloride, bromide, and iodide, and whose occupancy appears to be required for transport, was termed site II. Another site, which is less specific, binds a variety of monovalent anions and was termed site I. Binding of the respective anions to these sites can be detected by small but characteristic shifts in the absorption band of halorhodopsin, a red shift in the case of anions which bind to site II (Ogurusu et al., 1982; Steiner et al., 1984; Schobert et al., 1986), and a blue shift in the case of anions which bind to site I (Schobert et al., 1986).

The chromophore of halorhodopsin (absorption maximum at 565–578 nm) is in a pH-dependent equilibrium with a species containing a deprotonated Schiff base, whose absorption maximum is near 410 nm (Lanyi & Schobert, 1983; Steiner et al., 1984; Hegemann et al., 1985; Schobert et al., 1986). Occupancy of site I by various anions raises the pK_a of the Schiff base by up to 2 pH units, as detected either by a shift of the equilibrium in favor of the protonated species at a pH near the pK_a (Lanyi & Schobert, 1983; Steiner et al., 1984) or by a shift of the pH titration curve of the absorption at 570 nm toward higher pH (Schobert et al., 1986). Quantitation of the dependency of the apparent pK_a on anion

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concentration ruled out the model where site I is the positively charged Schiff base nitrogen, i.e., where the anions bind directly to the Schiff base (Schobert et al., 1986). Rather, the results favored the model where site I is a binding site distinct from the Schiff base, and the anion influences the deprotonation of this group upon binding to site I by direct (perhaps electrostatic) interaction. Such interaction of the Schiff base and the bound anion is consistent with the direction of the wavelength shift observed when anions bind to site I, because a blue shift is expected on theoretical grounds when a negative charge is introduced in the neighborhood of the Schiff base of a retinal (Honig et al., 1979; Warshel, 1979; Warshel & Ottolenghi, 1979; Kakitani et al., 1985).

In this report, we explore the nature of the interaction of the anions with site I and with the Schiff base. Since this interaction appears to be entirely, or nearly entirely, of an electrostatic nature, we are able to comment on the location of site I and on the nature of the electric coupling between the Schiff base and the anion at site I. The results suggest that halorhodopsin might be capable of an allosteric-type conformational change, which allows the efficient transfer of the binding energy at site I to the Schiff base.

Materials and Methods

Halobacterium halobium, strain OD-2, was grown in a peptone medium, as described before (Lanyi & MacDonald, 1979). Halorhodopsin was purified according to Steiner and Oesterhelt (1983), with minor modifications. The Lubrol was exchanged on a phenyl-Sepharose column for 1% nonanoyl-*N*-methylglucamide (Hildreth, 1982) in 2 M NaCl/20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] pH 6.0, and this detergent was then removed by dialysis. The samples were stored in 2 M NaCl, 20 mM MES, pH 6.0, and 0.1% octyl glucoside in the dark at 4 °C, where they were stable for months. Since we always dialyzed the samples to replace the buffer before use, little or no detergent was present during the experiments. All experiments were done at 15 °C where halorhodopsin was more stable than at room temperature.

Determination of the Binding Affinity for Anions. One milliliter of the halorhodopsin solution (5 nmol/mL) was dialyzed for 4 h against 2 × 500 mL of 0.5 M Na_2SO_4/50 mM EPPS, pH 8.2 at 4 °C. The titration was in the dark, with small volumes of a 4 M solution of the sodium salt of the anion in question, followed by determination of the absorption spectrum (in a Gilford Model 2600 single-beam spectrophotometer) after each addition. The display scale was corrected for the dilution of the samples by the additions.

Determination of Anion-Dependent Changes in the pK_a of the Schiff Base. These determinations were spectroscopic titrations in the dark with NaOH, as described elsewhere (Schobert et al., 1986).

Results and Discussion

We tested the binding of a series of monovalent anions to site I by determining the concentration dependence of the effect of the anions on the deprotonation equilibrium [as in Steiner et al. (1984)]. Plots of the absorbance increase at near 570 nm vs. the anion concentrations yielded apparent dissociation constants for the anions. Figure 1 shows a Lineweaver–Burk plot constructed from data for several anions. It is clear from Figure 1 that site I shows a distinct specificity, and the dissociation constants, K_1, range from about 5 mM (for bromide) to 126 mM (for chlorate). The dissociation constants calculated in this way do not seem to be affected by any chaotropic effects or oxidation of the protein by some of the anions used, because all plots, such as in Figure 1, were linear over large concentration ranges.

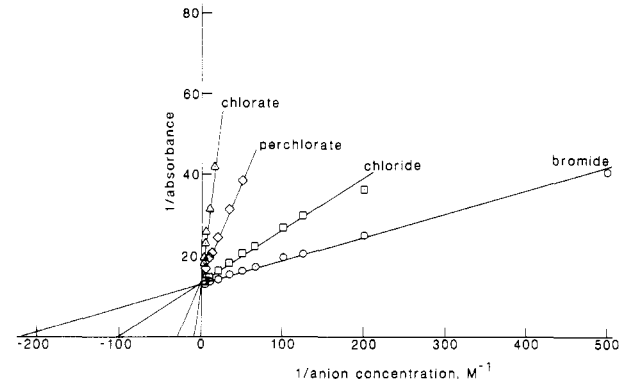

FIGURE 1: Lineweaver–Burk plot of the effect of various anions on halorhodopsin absorbance in the 565–575-nm region. Halorhodopsin (5 nmol/mL) was in 0.5 M Na_2SO_4/50 mM EPPS, pH 8.2. The intercepts of the plots on the x axis yield apparent dissociation constants for site I anion binding, K_1.

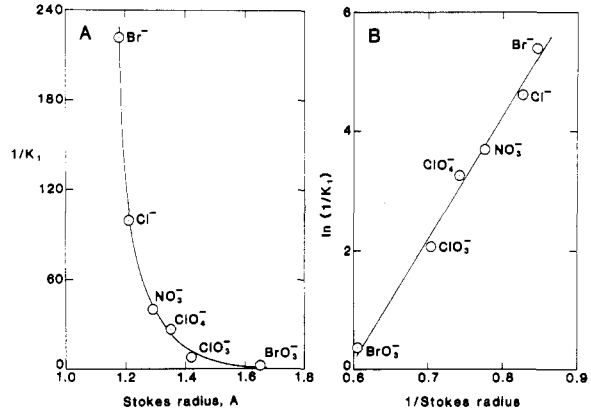

FIGURE 2: Dependence of the affinity of site I for various anions on the Stokes radii of the anions. (A) The association constants (reciprocals of the dissociation constants, K_1, from experiments as in Figure 1) are plotted vs. the Stokes radii, r (in angstroms). (B) The logarithms of the association constants are plotted vs. the reciprocals of the Stokes radii, $1/r$, consistently with eq 2. Stokes radii from Nightingale (1959).

We find that the association constants ($1/K_1$) obtained in this way are related to the Stokes (i.e., hydrodynamic) radii of the anions. As shown in Figure 2A, the affinity of site I to six anions for which estimates of Stokes radii are available (Nightingale, 1959), Br^-, Cl^-, NO_3^-, ClO_4^-, ClO_3^-, and BrO_3^-, expressed as the association constant, decreases steeply with the Stokes radius, r, and approaches zero when the latter exceeds 1.65 Å. This finding explains why some anions were found earlier to have no effect on the deprotonation equilibrium of halorhodopsin (Steiner et al., 1984), e.g., fluoride with r = 1.66 Å and sulfate with $r \approx 2.30$ Å. No such consistent relationship of the binding constant with crystallographic or hydrated radii was observed. Remarkably, the quantity ln ($1/K_1$), which is proportional to the interaction energy between site I and the anion [$\Delta G = -RT \ln (1/K_1)$], is linearly dependent on $1/r$ for all of the anions tested (Figure 2B). If the interaction between site I and the anion is approximated by Coulombic attraction between the two charges, the interaction energy will depend on the reciprocal of the distance between site I and the anion, taken as point charges. However, such use of Coulomb's law would predict a dependency in Figure

[1] Abbreviations: EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

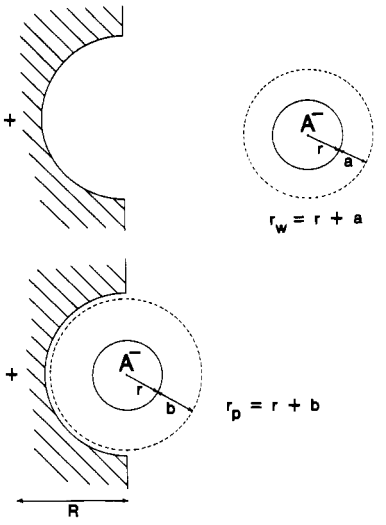

FIGURE 3: Schematic model of the interaction of anions with water in the free state (upper scheme) and with solvating groups of the protein when bound to site I (lower scheme) in halorhodopsin. A^- is the anion, (+) is the positive charge associated with site I, r is the Stokes radius, and R is the distance between the center of the anion and site I. The areas enclosed by dashed lines indicate the dimensions of the cavities around the anions inside their solvation shells. The radii of these (Born radii) become larger when the anions bind to the protein because of less effective solvation: radius in water, $r_w = r + a$; after binding, $r_p = r + b$, in eq 2.

2B on $1/(r + x)$, where x is the distance of closest approach of the anion to site I. Realistic values of $r + x$ are in the range of at least several angstroms, precluding linearity with $1/r$, as found in Figure 2B. A more sophisticated model for the interaction of an external charge with a protein is one used by Warshel and Russell (1984), in which they consider the Born self-energies of the interacting charges. In our case, for anion binding to site I, it is the free energy difference, ΔG, between the protein–anion complex and the protein, with the hydrated anion removed to infinite distance, which must be calculated. The two states are sketched in Figure 3. Using Figure 2B and eq 81 in Warshel and Russell (1984), for two single charges

$$\Delta G = -332/\epsilon_R R + (166/r_w)(1 - 1/\epsilon_w) - (166/r_p)(1 - 1/\epsilon_p) \quad (1)$$

where ϵ_R is a distance-related parameter, R is the distance between site I and the anion, r_w and r_p are the radii of the cavities the ion finds itself in when solvated by water and when solvated by groups furnished by the protein (Born radii), respectively, and ϵ_w and ϵ_p are equivalent to the local dielectric constants in water and in the protein. The second and third terms are the Born energies of the anion when in water and when bound to the protein, respectively, and the first term represents the interaction of site I with the anion, expressed as Coulomb's law with ϵ_R as a suitably chosen constant. If ϵ_w and ϵ_p are $\gg 1$, as seems likely in the vicinity of the ion both in water and at the protein, and the radii are written as $r_w = r + a$ and $r_p = r + b$ (where r is the Stokes radius)

$$\Delta G = -\frac{332}{\epsilon_R R} - 166\left(\frac{1}{r+b} - \frac{1}{r+a}\right) \quad (2)$$

Thus, the Coulombic attraction between the charges is partly compensated for by the change in Born energy from the dehydration of the ion upon binding to the protein. Equation 2 is represented by the line drawn in Figure 2B, with $-332/\epsilon_R R = 17$ kcal/mol, $a = 1.1$ Å, and $b = 2.3$ Å. Thus, the observed dependency on r, i.e., the preference of site I for anions with smaller Stokes radii, appears to come from the rather large change in the Born energy of the anion upon binding, rather than from the Coulombic term. The increase in Born radius upon binding (illustrated in Figure 3) reflects the less effective solvation of the anion by groups furnished by the protein than by water dipoles. The Born radii which we obtain seem to be reasonable, at least for the free anions where their value can be estimated by other means. Thus, for example fitting the data to eq 2 gives for chloride a Born radius $r_w = r + a = 2.3$ Å, which gives a predicted solvation energy of $166/2.3 = 72$ kcal/mol. The observed value is 79 kcal/mol (Friedman & Krishnan, 1973). The distance between site I and the anion, R, could be estimated if ϵ_R were known; the maximal value of R is about 4 Å for the lowest reasonable ϵ_R, which is 5. Site I is therefore within a few angstroms from the surface of the protein.

We report elsewhere (Schobert et al., 1986) that the binding of chloride or nitrate to site I causes an increase in the pK_a of the retinal Schiff base. In this study, we found that the magnitude of the maximal ΔpK_a was different for each anion and investigated this parameter in order to describe the interaction of anions bound to site I with the Schiff base. Shifts in the pK_a of protonated residues in response to neighboring charges have been analyzed by investigators for a number of proteins. For example, the effect of anion binding on the ionization of two histidine residues at the active site of ribonuclease was described by Matthew and Richards (1982), who showed that with increasing anionic charge of the protein the pK_a of these residues increased by up to 2 pH units. Warshel (1981) found that the pK_a of a glutamate residue at the active site of lysozyme was raised by a nearby ionized aspartate residue. These effects, and others where one of the charges is an external ion, are accounted for by a Coulomb-type interaction between the neighboring charged groups. Warshel et al. (1984) emphasized that the effective dielectric constant in such interactions is high because point charges in proteins must be surrounded by dipoles supplied by the protein residues, similarly to the case of free ions where water of hydration serves this function. Hence, for single interacting charges, ΔpK_a is usually less than 1 and seldom as high as 2. The change of the energy of deprotonation is given by $\Delta\Delta G_{deprot} = -2.3\ RT\ \Delta pK_a$.

In a series of experiments, the ΔpK_a was determined, as described elsewhere (Schobert et al., 1986), in the presence of each anion shown in Figure 2, at four to five different concentrations. Lineweaver–Burk plots were drawn for each anion to obtain the maximum ΔpK_a at infinite anion concentraton (not shown). These ΔpK_a values are plotted vs. r in Figure 4A. It is seen in Figure 4A that ΔpK_a, like $1/K_1$, is a steep function of the Stokes radii of the bound anions and the magnitude of the ΔpK_a for chloride and bromide is very large compared to what is observed in other systems. If $2.3\Delta pK_a$ is plotted vs. the reciprocal of the Stokes radius (Figure 4B), a line is obtained whose slope is similar to that of the line describing the depencence of the binding energy on the Stokes radii (cf. Figure 2B). This is unexpected and suggests a close relationship between anion binding and the effect of the binding on the Schiff base. The energies of binding for the anions, in fact, agree remarkably well with the changes in the energy of deprotonation upon the binding, as seen in Figure 5, where the slope of the relationship between these two quantities is about 0.85. The following alternatives might explain this unexpected result: (a) the electrostatic interaction between the anion and the Schiff base is large and

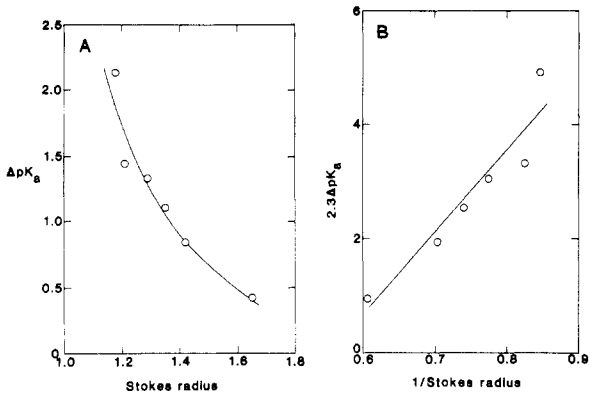

FIGURE 4: Dependence of the ΔpK_a, caused by binding of anions to site I, on the Stokes radii of the anions. The maximal values of ΔpK_a were determined by extrapolation of the ΔpK_a measured to infinite anion concentrations, for the anions shown in Figure 2. Stokes radii in angstroms, as in Figure 2. (A) Plot of ΔpK_a vs. the Stokes radius. (B) Plot of $2.3\Delta pK_a$ vs. the reciprocal of the Stokes radius, analogously to Figure 2B.

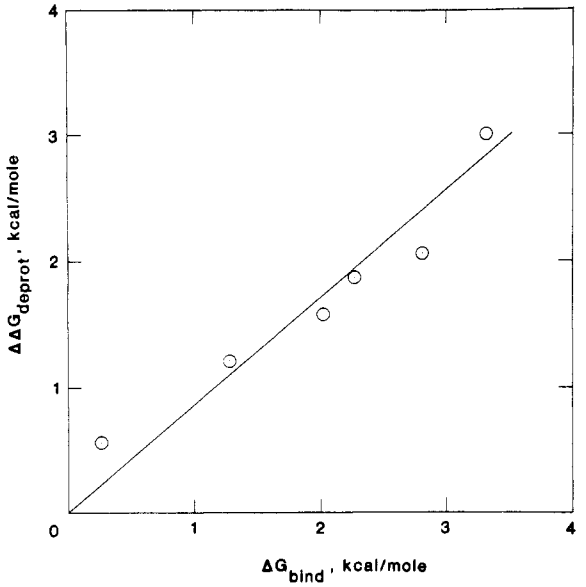

FIGURE 5: Correlation of the change in deprotonation energy of the Schiff base, $\Delta\Delta G_{deprot}$, upon anion binding to site I with the binding energy at site I, ΔG_{bind}. ΔG_{bind} and $\Delta\Delta G_{deprot}$ were calculated from the data in Figures 2B and 4B.

about equal to the binding energy because the positively charged Schiff base is itself site I; (b) the distance between the Schiff base and the bound anion is small and the electrostatic interaction is large because the Schiff base is in the immediate vicinity of site I; or (c) the distance between the Schiff base and the bound anion is not necessarily small, but anion binding to site I causes a conformational change in the protein which leads to the observed large ΔpK_a. According to possibility a, the Schiff base constitutes site I, but this model was ruled out elsewhere on kinetic grounds (Schobert et al., 1986). Possibility b is realized only if the distance between site I and the Schiff base is short enough to allow effective interaction between the bound anion and the protonated nitrogen. According to this alternative, the Schiff base is near the protein surface, as is site I. However, the observed highly efficient energy transfer (Figure 5) would not be possible without postulating an unrealistically short distance for a Coulomb-type interaction, even with an effective dielectric constant as low as 5. This leaves possibility c, which states in effect that the binding of the anions to site I is accompanied by displacement of protein (or perhaps anion) permanent dipoles along the electric field vector without significant reorientation; i.e., the binding energy is transmitted without large losses to the Schiff base. A conformational change leading to such a long-distance interaction has been postulated to take place in allosteric enzymes upon ligand binding, but direct evidence in support of this kind of mechanism is generally lacking.

The dependency of the ΔpK_a on the Stokes radii of the bound anions thus yields the interesting suggestion that the Schiff base in purified halorhodopsin is strongly coupled to a specific anion binding site on the protein surface, perhaps by an allosteric-type mechanism. It seems likely that this is the case for membrane-bound halorhodopsin also, because the effect of chloride on the pK_a of the Schiff base of halorhodopsin in cell envelope vesicles, consistent with the concept of site I, is well documented (Lanyi & Schobert, 1983). Although the data for site I and its interaction with anions are incomplete in this system, neither the affinity of site I for chloride nor the magnitude of the increase in pK_a is contradictory to the data with the purified pigment. Since the spectroscopic changes associated with binding to site I in the vesicles were observed instantaneously after adding NaCl, while the permeability of the membrane to chloride is rather low (Schobert & Lanyi, 1982), site I must be on the extracellular side of the vesicle (and cell) membrane, i.e., the side from which the chloride is transported. The possible involvement of site I in the chloride transport is, of course, an intriguing possibility.

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Registry No. Chlorate, 14866-68-3; perchlorate, 14797-73-0; chloride, 16887-00-6; bromide, 24959-67-9.

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Investigation of Polylysine–Dipalmitoylphosphatidylglycerol Interactions in Model Membranes†

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ABSTRACT: The effect of poly(L-lysine) on dipalmitoylphosphatidylglycerol bilayers has been studied by Raman and infrared spectroscopies, small-angle X-ray diffraction, and carboxyfluorescein escape experiments. The polypeptide is shown to induce a stabilization of the bilayer detected by the increase of interchain vibrational coupling and a slight decrease of the overall disorder. In addition, long polylysine (M_r 150000) induces a positive shift of the gel to fluid transition temperature and, at lipid to lysine molar ratios greater than 1, a lateral phase separation within the bilayer. Raman and infrared spectra indicate modifications at the head group level. In contrast, short polylysine (M_r 4000) leads to a decrease of the lipid thermotropic transition temperature, and no modification of the polar head group and no phase separation could be observed. These differences between short and long polypeptides are correlated with the conformation the polypeptide adopts upon binding to the lipid, which favors the formation of α-helices in the case of long polylysines (M_r $\geq$14000). The X-ray data suggest that the basic polypeptide acts as a bridge between neighboring bilayers, thus causing their aggregation and dehydration.

The function of biological membranes is believed to be intimately related to their structure, which in turn strongly depends on the interactions between their main constituents, namely, lipids and proteins. In this study, we focused on a specific model system that mimics the complex formed by a peripheral protein and the lipidic surface. The binding of such proteins is primarily driven by electrostatic forces arising between their exposed positively charged residues and some negatively charged lipids of the bilayer. Owing to its strongly basic character, poly(L-lysine) (named PLL[1] hereafter) is ideally suited to model an extrinsic protein. The acidic lipid dipalmitoylphosphatidylglycerol (DPPG) was used in order to maximize the electrostatic interactions.

Polylysine has an unordered conformation at neutral pH but can form either α-helices or β-sheets in alkaline solution. In a circular dichroism study, Hammes and Schullery (1970) showed that PLL (M_r 100000) takes the α-helical conformation when it binds to natural phosphatidylserine, at neutral pH. The same effect was observed by Raman spectroscopy (Carrier & Pézolet, 1984) for dipalmitoylphosphatidylglycerol and PLL (M_r 150000). However, it was recently proposed (Carrier et al., 1985) that polylysines with lower degree of polymerization may fail to adopt such an ordered conformation upon binding to DPPG bilayers, because they did not lead to the same complex modifications of the lipid thermotropic behavior. Long poly(lysines) (M_r $\geq$60000) were shown to induce the formation of three distinct types of domains when there was less than one lysine residue per lipid molecule (R_i > 1). Our first aim was thus to verify if such lateral phase separation may be seen by Raman spectroscopy and to determine the conformation of bound polylysine of low molecular weight.

Many authors suggested that the binding of PLL to acidic lipids might also involve hydrophobic forces in addition to the straightforward electrostatic interactions. While purely electrostatic bonds are destroyed at high ionic strength, high salt concentrations (1 M NaCl) failed to completely disrupt PLL/phosphatidylserine complexes (Hammes & Schullery, 1970). From EPR spectroscopy, Hartmann and Galla (1978) concluded from the decrease of the hyperfine coupling constant of labeled polylysine that the side chains of this polypeptide penetrates into the bilayer. Shafer (1974) found that PLL exhibits a two-step mechanism of interaction with phosphatidylserine monolayers: the initial binding produces a decrease of the surface pressure, most likely due to the neutralization of lipidic head groups, followed by a gradual positive change in surface pressure that was interpreted as a consequence of the entry of the lateral side chains of the polypeptide into the lipid monolayer. This evidence that polylysine can act as a spacer between the lipidic polar heads led us (Carrier & Pézolet, 1984) to suggest the possiblity of PLL-induced interdigitation of DPPG bilayers in order to explain the change

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[1] Abbreviations: PLL, poly(L-lysine); DPPG, dipalmitoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; CF, carboxyfluorescein; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; T_c, gel to liquid-crystalline phase transition temperature; IR, infrared; E_a, activation energy.

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