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PEPTIDE-MEMBRANE INTERACTIONS

A FLUORESCENCE STUDY OF THE BINDING OF OLIGOPEPTIDES CONTAINING AROMATIC AND BASIC RESIDUES TO PHOSPHOLIPID VESICLES

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The binding of oligopeptides containing basic and aromatic residues to phospholipid vesicles has been studied by fluorescence spectroscopy. Tryptophan-containing peptide such as Lys-Trp-Lys or Lys-Trp(OMe) exhibit a shift of their fluorescence toward shorter wavelengths and an increased fluorescence quantum yield upon binding to phosphatidylinositol (PI) or phosphatidylserine (PS) vesicles. No binding was detected with phosphatidylcholine vesicles. The binding is strongly dependent on ionic strength and pH. Binding decreases when ionic strength increases indicating an important role of electrostatic interactions. The pH-dependence of binding reveals that the apparent p*K* of the terminal carboxyl group of Lys-Trp-Lys is raised by ~3 units upon binding to PI and PS vesicles. The binding of tyrosine-containing peptides to PI and PS vesicles is characterized by an increase in the fluorescence quantum yield of the peptide without any shift in fluorescence maximum. A natural nonapeptide from the myelin basic protein which contains one tryptophan residue binds to PI and PS vesicles at low pH when the acidic groups are neutralized. This binding is accompanied by a shift of the tryptophyl fluorescence toward shorter wavelengths together with an enhancement of the fluorescence quantum yield. Dissociation of the complex is achieved at high ionic strength. These results indicate that aromatic residues of oligopeptides bound to the phospholipid polar heads by electrostatic interactions become buried in a more hydrophobic environment in the vicinity of the aliphatic chains of the lipids.

1. Introduction

There have been several attempts to use polypeptides to mimic the lipid-protein interactions that occur with intrinsic and extrinsic membrane proteins, and with proteins that act on membranes. These include polylysine [1,2], copolymers such as $(Lys\text{-}Phe)_n$ or $(Lys–Tyr)_n$ [3] as well as poly(Ala-Lys) [4] or poly-(Leu-Trp) [5] which have been syntehsized as models of amphipathic or intrinsic proteins. Another approach to explore these problems is to define and isolate peptides involved in protein-membrane interactions. Experiments with protein fragments have been carried out with the hydrophobic part (T_{is}) of glycophorin [6], that of cytochrome b_5 [7], peptides related to apolipoproteins [8], pentagastrin [9] and the basic protein from myelin [10].

In many cases, it has been concluded that electro-

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Abbreviations: EAE, experimental allergic encephalitis; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; KWK, lysyltryptophanyllysine; KW(OMe), lysyltryptophanyl, methyl ester; KYK(NHEt), lysyltyrosyllysinethylamide.

static forces could trigger such binding, but conclusions are obscured by superimposition of many different contributions. Simple peptides should allow us to analyze the different types of molecular interactions which are involved in the association of proteins with the phospholipid constituents of membranes. In order to achieve maximum simplification, we have studied interactions of phospholipid vesicles with di- and tripeptides, associating lysine, often implicated in the interactions at the interface, with tryptophan or tyrosine, which are among the most hydrophobic residues. Experiments done with a peptide from the myelin basic protein illustrate the relevance of such interactions for natural peptides. The changes in the intrinsic fluorescence of these peptides have been used to follow lipid-peptide interactions.

2. Material and Methods

Lysyltryptophanyllysine (KWK) was purchased from Bachem and Serva Feinchemicals and used without further purification. Lysyltryptophanyl methyl ester (KW(OMe)) and lysyltyrosyllysinethylamide (KYK(NHEt)) were synthesized by Drs. J. Rossi and R. Mayer (Orléans), respectively. The nonapeptide from the myelin basic protein was from Serva. Egg phosphatidylcholine (PC) was prepared by M. Charlier and R. Bernon according to Singleton et al. [11]. Brain phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol (PG) were obtained from Lipid Products, Nutfield, U.K. Phospholipids were dispersed either in phosphate (20 mM) or Tris-acetate buffer (20 mM) at pH 7.5 and sonicated to obtain unilamellar vesicles [12]. When binary lipid mixtures were used, PC and PS were mixed in organic solvent in appropriate amounts and dried. The homogenous lipid mixture was then dispersed in buffer and sonicated as described above.

Fluorescence experiments were carried out at 25°C on a FICA 55 MK differential spectrofluorometer which automatically corrected the emission spectra. Excitation wavelength was usually 280 nm.

The ionic strength was varied by adding small amounts of a 4 M KCl solution to the cuvette. The pH was changed with 1 M NaOH or HCl and measured directly in the sample. The changes observed upon decreasing pH were always reversible. However when the pH was increased no reversibility was found above pH 10.

3. Results and Discussion

3.1. Binding of tryptophan-containing peptides to lipid vesicles

At pH 7.5 the addition of negatively charged phospholipids such as PS and PI resulted in a blue shift of the emisssion spectrum of KWK (Fig. 1). A smooth variation was observed with no plateau being reached in the range of lipid-to-protein molar ratio (R_i) investigated. This indicated that KWK interacted with the negatively charged vesicles but that the association constant was not very high since it was not possible to obtain total binding of the peptide in the

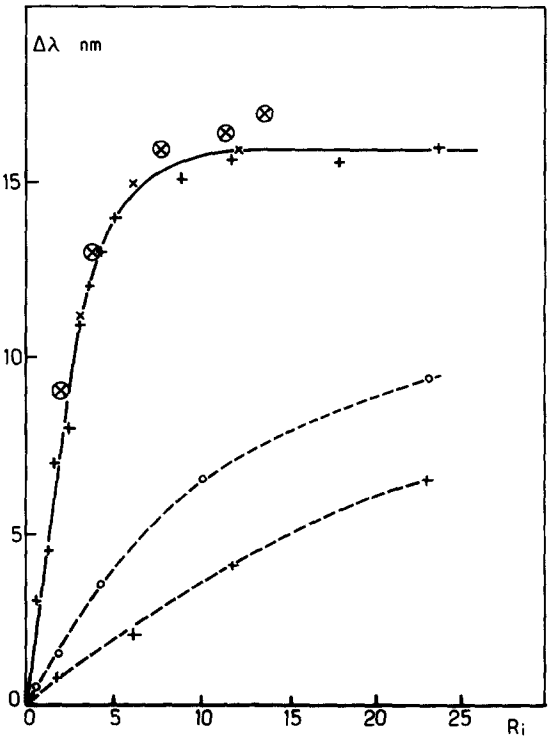

Fig. 1. Changes in wavelength (Δλ) of the fluorescence maximum of KWK in the presence of increasing amounts of phospholipid vesicles. R_i is the lipid-to-peptide molar ratio. Dotted lines correspond to the interaction at pH 7.5 of KWK (50 μM) with PS (o- - - - -o) and to that of KWK (5 μM) with PI (+- - - - -+). Full line corresponds to the interaction of KWK at pH 3.5 with PI at different peptide concentrations: 1.5 μM (×), 5 μM (+) and 10 μM (⊗).

concentration range around 10^{-5} M.

In the case of PC vesicles no significant change in fluorescence was observed with KWK. A small shift (2–3 nm) of the fluorescence maximum was observed at high R_i values. For binary PC-PS mixtures the capacity to bind KWK increased with the PS content. As shown on Fig. 2, the shift of the emission maximum increased up to a value around 13 nm and concomitantly the quantum yield increased.

Similar experiments were carried out at pH 3.5 when the terminal carboxylic group of the peptide was protonated. As it can be seen on Fig. 1, the interaction of KWK with PI vesicles was enhanced and led to stoichiometric binding curves that were identical for peptide concentrations ranging from 1.5 to 25 μM. This result indicated that the association constant was at least of the order of 10^7 M^{-1}. An extrapolation of the linear part of the binding curve (low R_i) allowed us to estimate that the minimum number of lipids required for each peptide was about 4.

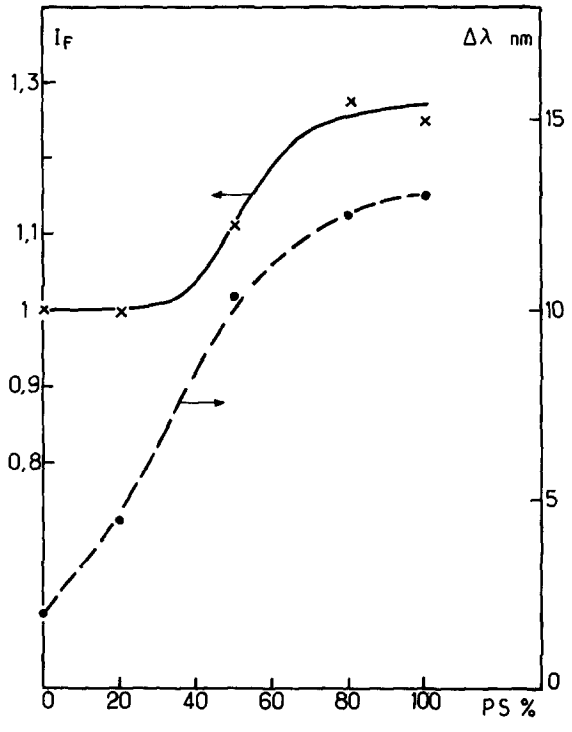

Fig. 2. Changes in wavelength (Δλ) of the emission maximum (•------•) and in relative quantum yield (x——x) of KWK in the presence of mixed PC-PS vesicles. R_i = 107 [KWK] = 10 μM, pH = 7.5.

The important role of the carboxylic group was demonstrated by studying the interaction of phospholipids with the dipeptide KW(OMe) whose terminal carboxylic group was blocked by methylation (Fig. 3). The changes in fluorescence spectrum observed at pH 7.5 were very similar to those described above with KWK at pH 3.5. Furthermore an isoemissive point was observed at 385 nm (Fig. 3a) indicating that along the binding process only two different fluorescent states (free and bound) of the peptide have to be considered. No change was observed in the case of PC vesicles (Fig. 3b). A plot of Δλ (the shift of fluorescence maximum) versus R_i (ratio of phospholipid to peptide concentration) reached a plateau which did not depend on KW(OMe) concentration in the range 1.5 to 17 μM indicating that the association constant is at least of the order of 10^7 M^{-1}. The stoichiometry is about 4 phospholipids per oligopeptide, a value identical to that found above with KWK (Fig. 1).

Experiments carried out at pH 10, when KW(OMe) bears a single positive charge on the lysine residue, indicated that the affinity for negatively charged lipids was decreased, but that binding still occurred (Fig. 3b).

3.2. Effect of ionic strength on the stability of the complexes

Adding KCl to the complexes formed at low salt concentration restored the fluorescence characteristics of the free peptide. Therefore when the surface potential is decreased the tryptophan residue is released from the interface. For PS vesicles at pH 7.5 half dissociation occured at 50 mM and 300 mM KCl for KWK and KW(OMe), respectively. In the case of mixed vesicles of various PC-PS content less than 5% of the peptide remained bound in 150 mM KCl.

3.3. Effect of pH on the stability of the complexes

The fluorescence intensity of Trp-containing peptides is known to vary with pH [13]. However the wavelength of the emission maximum remains practically unchanged [14]. Therefore, the changes reported on Figs. 4a and 4b are directly related to the association-dissociation of peptide-lipid complexes. On

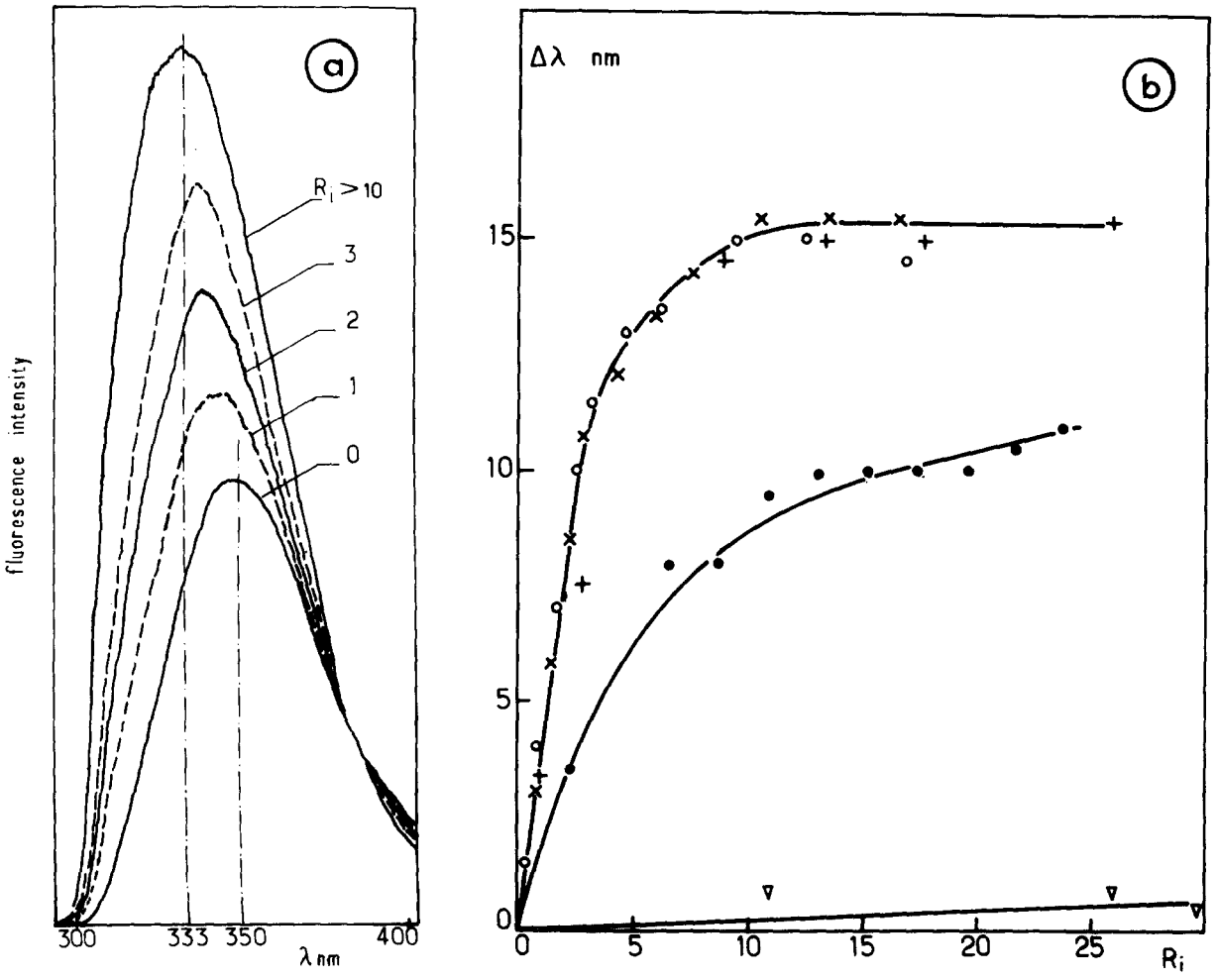

Fig. 3. Changes in the emission spectrum of KW(OMe) in the presence of increasing amounts of phospholipid vesicles. (a) Fluorescence spectra at different R_i values at pH = 7.5, 20 mM Tris acetate, [KW(OMe)] = 17 μM. (PI vesicles). (b) Changes in wavelength (Δλ) of the fluorescence maximum versus R_i. (○) PS + 17 μM KW(OMe), pH 7.5; (×) PI + 17 μM KW(OMe), pH 7.5; (+) PI + 1.5 μM KW(OMe), pH 7.5; (▽) PC + 17 μM KW(OMe), pH 7.5; (●) PS + 28 μM KW(OMe), pH 10.

the low pH side it can be seen that complexes are dissociated with pK values around 3.5 for PS and 1.5 for PI in quite good agreement with the pK values of the carboxylic group of PS [15,16] and the phosphate group of PI [17], respectively. Similar results were obtained with KWK and KW(OMe) indicating that the terminal carboxylic group of the former was not involved in the observed transition even though its pK in the free peptide is around 3.2 [18]. A comparison of Figs. 4a and 4b leads to the conclusion that the carboxylic group of KWK is titrated around pH 6.5; KW(OMe) which lacks this carboxylic group does not show any variation of Δλ up to pH 7.5. This might be related to the shift of apparent pK values due to the charged surface of the phospholipid vesicles as already observed in model systems [19,20]. It should be noted that PI has no titrable group above pH 3 and that any effect observed in the pH range 3–10 must be attributed to the titration of peptide groups. The transition observed with a midpoint around pH 8.5 (Fig. 4b) is therefore ascribed to the titration of the α-amino group of the peptide. In the

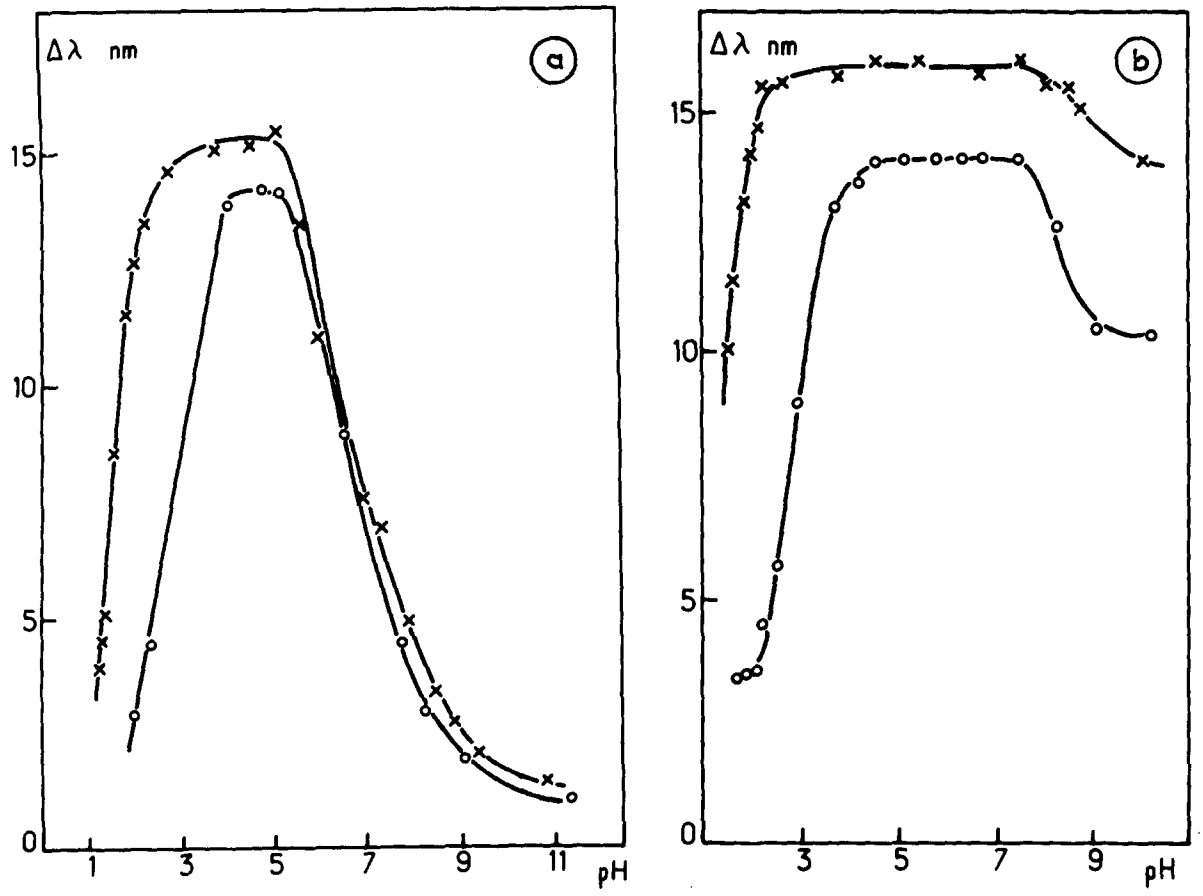

Fig. 4. pH dependence of the changes in wavelength (Δλ) of the emission maximum of peptides bound to PS (o———o) and PI (x———x). (a) [KWK] = 20 μM, [PS] = 330 μM (o); [KWK] = 28 μM, [PI] = 280 μM (x). (b) [KW(OMe)] = 24 μM, [PS] = 139 μM (o); [KW(OMe)] = 24 μM, [PI] = 168 μM (x).

free peptides (KWK and KW(OMe)) the p*K* of this group is around 7.5. The shift which is observed in the presence of phospholipids certainly results from electrostatic interactions as already observed in the complexes formed by the same peptides with nucleic acids [18].

In the case of PS one might have expected to observe an enhancement of KWK-lipid association due to an electrostatic interaction between the negatively charged carboxylate of the peptide and the positively charged amino group of the lipid. The results shown in Fig. 4a indicate on the contrary that ionization of the carboxylic group induces a dissociation of the peptide-lipid complexes. This suggests that the global charge of the peptide in the electrostatic potential of the phospholipid surface is the main driving force for peptide binding. However since KWK and KW(OMe) have the same global charge at pH 7.5 the stronger binding of KW(OMe) might be related to the presence of the hydrophobic-COOMe group at the end of this peptide. This might enhance the hydrophobic contribution to peptide binding already evidenced by the shift of the indole fluorescence spectrum toward shorter wavelengths.

3.4. Interaction of KYK(NHEt) with phospholipid vesicles

The fluorescence quantum yield of tyrosine is known to be sensitive to the environment but its fluorescence maximum is not markedly affected. A

blocked tyrosine-containing peptide, KYK(NHEt), was used to investigate the changes induced by interaction with lipids. The spectra of the peptide alone and in the presence of PS vesicles are reported in Fig. 5a at pH 7.5. An increase in fluorescence intensity was observed without any change in the emission maximum wavelength. The relative change in intensity plotted versus R_i shows a saturation curve with a plateau which depends on the nature of the phospholipid but which does not change when the concentration of KYK(NHEt) increases from 9 to 17 μM. This last result indicates that the binding constant is certainly higher than 10^6 M^{-1}. From the intial linear part of the binding curve the stoichiometry of KYK-(NHEt) for PS vesicles was estimated to be about 10 phospholipids per bound peptide.

3.5. Interaction of a natural peptide from the myelin basic protein

The nonapeptide Phe-Ser-Trp-Gly-Ala-Glu-Gly-

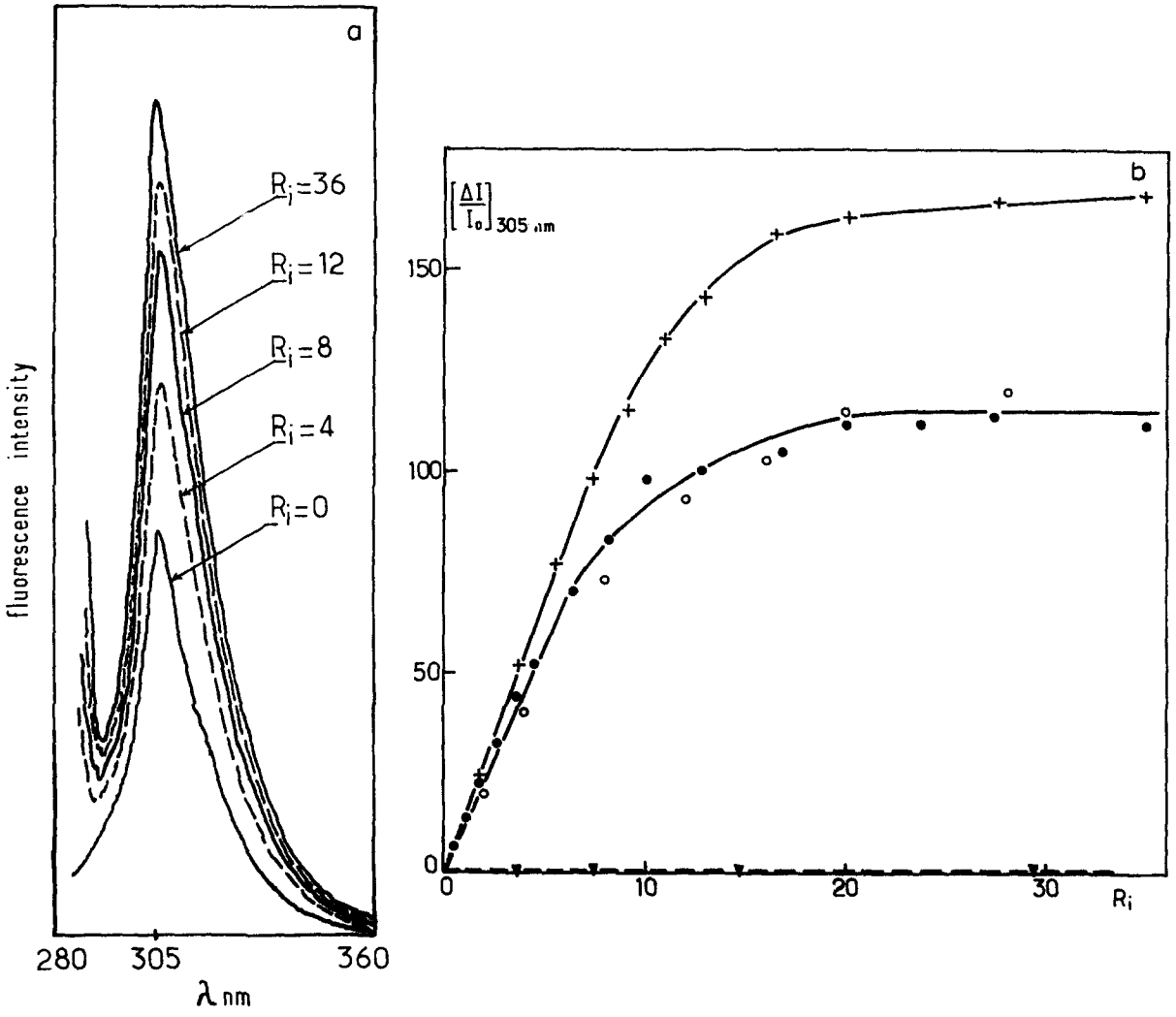

Fig. 5. Changes in the fluorescence of KYK(NHEt) in the presence of increasing amounts of phospholipid vesicles at pH = 7.5. (a) Emission spectra versus R_i in presence of PS, [KYK(NHEt)] = 9 μM. (b) Changes in the relative intensity at 305 nm ($\Delta I/I_0$) versus R_i. [KYK(NHEt)] ≈ 17 μM for PS (○), PI (+) and PC (▾) [KYK(NHEt)] = 9 μM with PS (●).

Gln-Arg is a tryptic fragment of the myelin basic protein. Like the whole protein it has the capacity to induce experimental allergic encephalitis (EAE) and it is therefore known as EAE peptide [21]. The peptide contains the single Trp of the whole protein. At pH 7.5 it has no net charge but at low pH values it bears positive charges (α-NH_3^+ and Arg). At pH 7.5, the fluorescence of the peptide is unchanged in the presence of charged and uncharged lipids (Fig. 6). However, at pH 3.5, changes in both the emission maximum and in fluorescence intensity are detected. The interaction occurs only with negatively charged

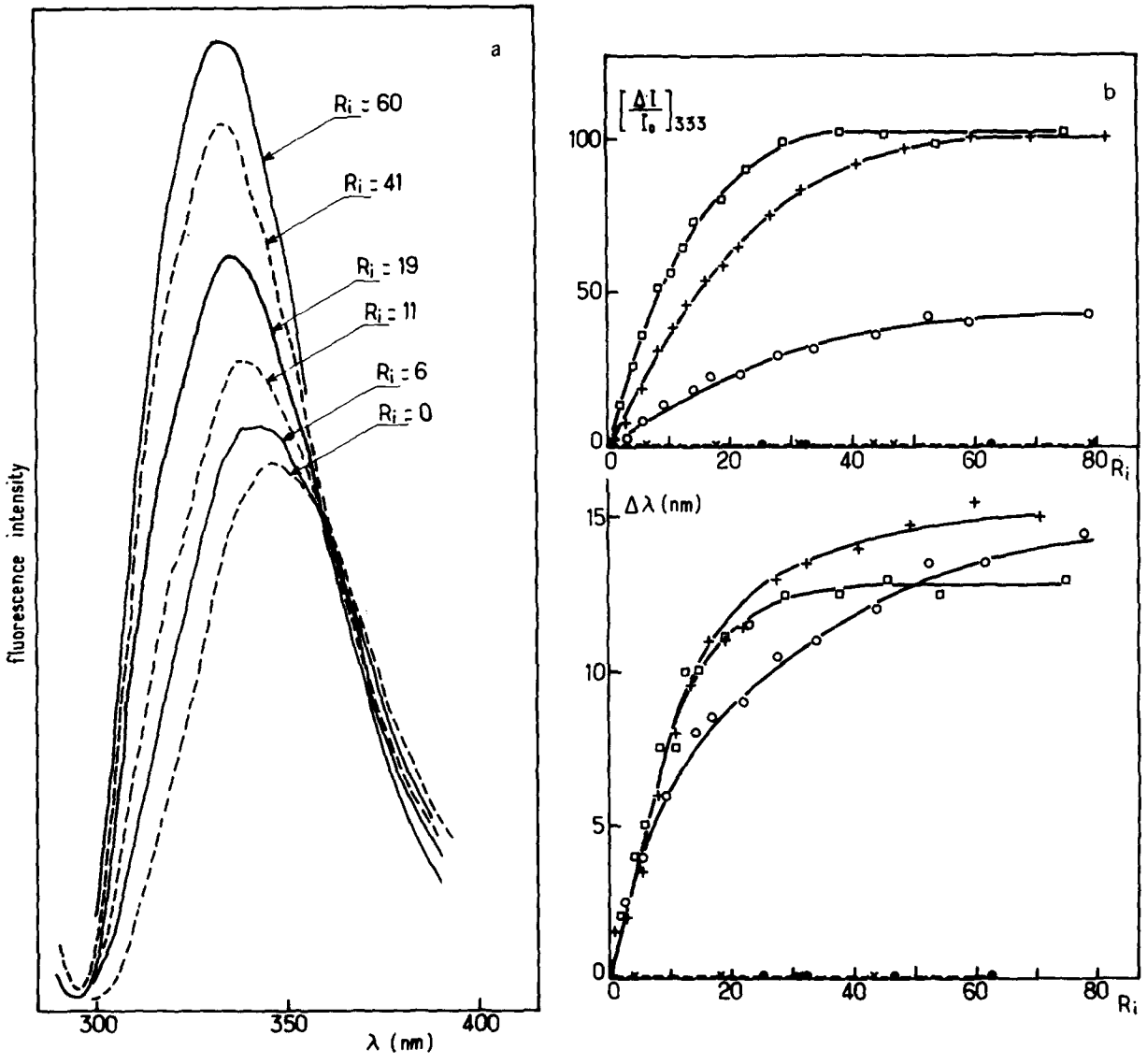

Fig. 6. Trp fluorescence changes of EAE nonapeptide in the presence of phospholipids vesicles. (a) Emission spectra at different PI to peptide molar ratio. R_i, [EAE] = 10 μM, Tris-acetate buffer 20 mM, pH 3.5. (b) Changes in the emission wavelength ($\Delta\lambda$) and fluorescence intensity at 333 nm ($\Delta I/I_0$) versus R_i, [EAE] = 10 μM. PS (○); PI (+); PG (□); at pH = 3.5. PS (●); PI (×) at pH 7.5.

lipids such as PS, PI and PG. The complexes are dissociated when the pH increases above 4.2; as in the case of synthetic tripeptides the addition of NaCl dissociates the complexes (data not shown).

4. Conclusions

The changes detected by intrinsic fluorescence indicate that di- and tripeptides interact with negatively charged lipids. This interaction is dominated by electrostatic forces since an increase in ionic strength or a decrease of the net positive charge of the peptide (increase in pH) induces a dissociation of these complexes, and since under similar conditions no significant binding occurs with PC vesicles. The changes observed in the emission parameters indicate that the tryptophan and tyrosine rings become buried in a less polar environment probably in the vicinity of aliphatic chains.

The interaction of such peptides with membranes could not be described as a simple equilibrium. The occupancy of the interface by the peptide decreases the surface charge of the vesicles and therefore the affinity decreases. Such a situation has been already observed for the interaction of these peptides with nucleic acids [22]. A descirption including the surface potential changes has been proposed in the case of cations [16,23] and melittin [24] interacting with bilayers. Moreover the overlap of peptide binding sites should be taken into account. Each phospholipid polar head is a potential origin for the binding site of one peptide molecule. When one peptide is bound it eliminates several potential binding sites because it covers (and interacts) with more than one polar head group. This problem is equivalent in a two-dimensional space to that already described for a one-dimensional lattice [26].

The affinity of the investigated peptides is strongly decreased upon ionization of their terminal carboxylic group, even with PS vesicles. Furthermore although at pH 7.5 KWK and KW(OMe) bear the same net charge the blocked dipeptide has a higher affinity. This implies that the negative charge on the peptide is not able to interact with the ammonium charge of the serine group but on the contrary introduces repulsive interactions. On the other hand, the methylated carboxyl group of KW(OMe) could be involved in hydrophobic interactions together with the indole ring and this might increase KW(OMe) binding as compared with KWK.

The above results have to be kept in mind when using peptides obtained from proteolytic cleavage of proteins interacting with membranes. This is illustrated by the behavior of the EAE peptide at the interface. The affinity is almost totally governed by the complementarity of the charges borne by the peptide and lipids. This totally agrees with the results obtained by Deber and Young [10] by gel chromatography. It should be noted that as soon as electrostatic conditions are fullfilled an hydrophobic burying of the indole ring of Trp [3] takes place. Similar findings have been obtained with other natural peptides such as the chymotryptic peptide 1–19 of melittin [25]. and a tryptic peptide of cardiotoxins (unpublished results) which only interact with negatively charged lipids.

An interesting observation made with KWK is the shift of the pK of the carboxyl group when this peptide is bound to both PS and PI vesicles. Even if this group is not involved in any interaction (as expected with PS vesicles) the polyelectrolytic environment due to the charged phospholipid surface is expected to shift its apparent pK [20,27]. Due to the negative electrostatic potential ψ the local pH is decreased by $0.43e\psi/kT$ (where e is the protonic charge). We have observed that the apparent pK of the terminal carboxyl group of KWK increases from 3.1 (free peptide) to ≈6.5 (bound to PS or PI vesicles) at low ionic strength (20 mM). This is in agreement with the expected increase in local pH due to the negatively charged phospholipid surface.

The results obtained with di- and tripeptides could help clarify and solve more general problems encountered when investigating lipid-protein interactions.

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