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Cercospora beticola toxins. VII. Fluorometric study of their interactions with biological membranes

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Abstract

The interactions of two beticolins, *Cercospora beticola* toxins, and of their magnesium complexes with liposomes or plasma membrane were studied. The fluorometric pH titration curves of beticolins in liposomes and in plasma membranes reveal the presence of the dissociated form of beticolins. The concentration of the magnesium complex in these membranes increases at high pH. The partition coefficient of beticolin-1 on liposomes is 3-fold higher than that of beticolin-2 and the fluorescence of both compounds on liposomes is similar. The addition of magnesium to liposomes causes a 40-fold and 20-fold increase in the partition coefficient of beticolin-1 and -2, respectively, as a result of the interactions between membrane, magnesium and beticolins. Beticolins react to a ΔpH across the liposome membrane but the formation of the magnesium complex completely abolishes this effect.

Keywords: Beticolin; Liposome; Plasma membrane; Fluorescence; Magnesium complex

1. Introduction

Cercospora beticola is the causal agent of cercosporiose, the most important leaf disease of sugar beet [1]. Among the yellow toxins produced by *C. beticola*, one was known as CBT. Its biological activities have been extensively described [2–7]. Macri et al. [5] reported the inhibition of ATP-dependent proton translocation in microsomal vesicles by CBT and suggested a primary effect on ATPase(s) associated with plasma membrane. Blein et al. [6] have shown that the inhibition of plasmalemma ATPase by CBT is stronger at high pH and competitive with ATP-magnesium. This result suggests that the complex CBT-magnesium might be the active inhibitory form of the ATPase. Data concerning the physico-chemical properties and the structure of CBT have been published in the last twenty years [1,8] but remain scarce. From a *C. beticola* strain, we isolated several yellow toxins named beticolins in order to elucidate their structure [9–11]. On the basis of monocrystals we obtained the structure of beticolin-2 by X-ray diffraction [9,10]. The structure of beticolin-1 was deduced by comparing NMR and mass spectrometry data [9]. Recently, a compound called cebetin B [12,13] was isolated from *C. beticola* by two groups [12,13]. It is a dimeric chelate of cebetin A (cebetin A and beticolin-1 have the same structure).

We showed [14] that three of the six hydroxyl groups of beticolin-1 and -2 (Fig. 1) dissociated in 50% (v/v) dioxane-water solution. The dissociation of two groups took place at a physiological pH and the uncharged form of the magnesium complex was predominant in the solution at this pH range. The dissociation of the first hydroxyl group did not bring about any apparent changes neither in the absorption nor in the fluorescence spectra. The uncharged magnesium complex fluoresced in water and in 50% (v/v) dioxane-water solution.

Due to their hydrophobicity beticolins are concentrated in the membrane where they interact with a target enzyme. In the study of the interaction of beticolins with biological membranes, fluorometry is a very

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CBT, *Cercospora beticola* toxin; H_3B, HB^{2-}, the non-dissociated form and the dianion of beticolin.

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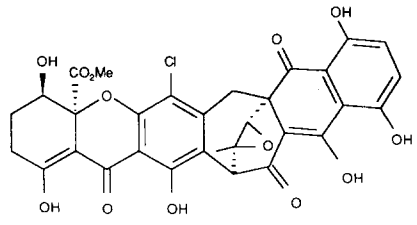
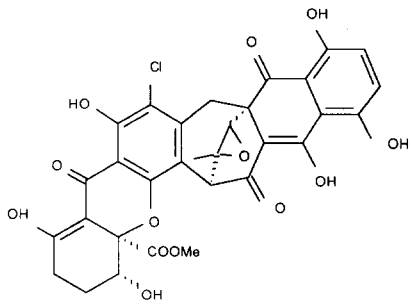


beticolin-2



beticolin-1

Fig. 1. Chemical structure of beticolin-1 and -2.

suitable method to show that the complex is the prevaling form of beticolins in membranes at physiological conditions.

2. Materials and methods

2.1. Materials

Corn root membrane vesicles were prepared according to Blein et al. [6]. The fraction of right-side out vesicles treated with Triton X-100 estimated on the basis of the ATPase activity before and after the permeabilisation with a detergent was about 50%. Beticolins-1 and -2 were purified from a mycelial extract of a *C. beticola* strain (CM) according to Milat et al. [9].

2.2. Preparation of liposomes

Liposomes were prepared by sonication (10 min) of a mixture of 10 mg soya phosphatidylcholine/ml and 10 mg sheepbrain phosphatidylethanolamine/ml in 20 mM Mes (pH 6.5, if not stated otherwise) under nitrogen. Next, the suspension was centrifuged (30 min, $13\,000 \times g$) in order to eliminate traces of non-dispersed phospholipids and the supernatant was used for the experiments.

2.3. Determination of the binding parameters

The binding parameters of beticolins were determined on the basis of the fluorescence increase after their binding on the membranes. A low beticolin concentration was titrated by an increasing concentration of membranes and double reciprocal plots $1/F$ versus $1/L$ were obtained as described by Mikes et al. [15]:

$$1/F = (1/(F_b P)) \cdot 1/L + 1/F_b$$

where F is the fluorescence, F_b the fluorescence of a bound compound (at the infinite membrane concentration), L the concentration of liposomes (mg/ml) or plasma membrane (mg protein/ml) and P the 'partition coefficient' (ml/mg). The partition coefficient P characterizes the affinity of the membrane for the compound and it is defined as $P = KN$ where K is the binding constant (ml/mmol) of the compound and N the maximal binding capacity (mmol/mg) of the membrane. The fluorescence of free beticolins in water is negligible and was not taken into the account. The plot of $1/F$ versus $1/L$ gave a straight line with a slope $1/(F_b P)$ and an intersection with the y-axis $1/F_b$.

The fluorescence spectra were recorded with a Shimadzu RF 5001 PC spectrofluorometer and the fluorescence intensities are expressed in arbitrary units.

3. Results and discussion

3.1. Interaction of beticolins with membranes

The interactions of beticolins with liposomes or plasma membrane were studied with beticolin-1 and -2 (Fig. 1). As their fluorescence properties are very similar only the data concerning beticolin-2 are presented. We first investigated the effect of the polarity on the fluorescence properties of beticolins. Fig. 2 shows the emission spectra of the non-dissociated form and of its dissociated form in a mixture dioxane-Mes (50 mM). The non-dissociated form in water showed excitation maxima at 340 and 440 nm, a low fluorescence and a broad emission maximum at 500 nm (data not presented). A decrease in polarity of the medium caused a blue shift of the emission maximum, which was at 470 nm in dioxane, and an increase in the fluorescence intensity. Similarly, the fluorescence of the dissociated form in water with excitation maxima at 350 and 455 nm and an emission maximum at 530 nm was very low. Fig. 2 shows that the fluorescence intensity of the dianion increased significantly in less polar medium and the emission maximum shifted to 507 nm in 80% (v/v) solution dioxane-Mes (50 mM). The effect of solvent polarity on the fluorescence properties of both forms of beticolin-2 proves that their dipole moments

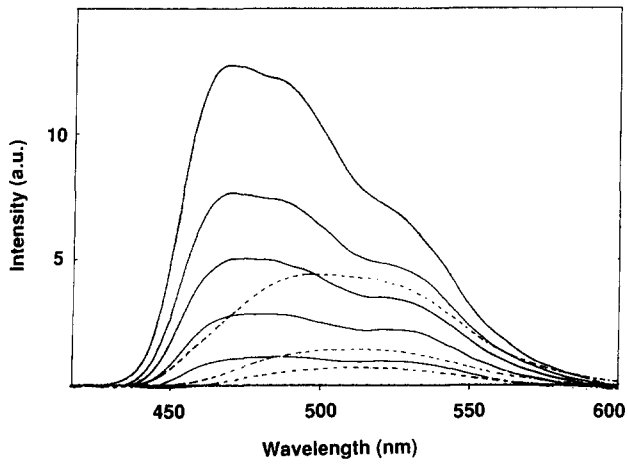


Fig. 2. Fluorescence emission spectra of the non-dissociated form (H_3B) and of the dianion (HB^{2-}) of beticolin-2 in a mixture (v/v) dioxane-50 mM Mes. Excitation wavelength was adjusted to 340 nm (H_3B) and to 350 nm (HB^{2-}). Full lines: 5.5 μM H_3B (pH 3.0). From the top to the bottom: 100%, 80%, 60%, 40% and 20% dioxane. Dashed line: 5.5 μM HB^{2-} (pH 7.2). From the top to the bottom: 80%, 60% and 40% dioxane.

in the excited state are higher than those in the ground state [16].

Biological membranes represent an environment of low polarity. Beticolins do not fluoresce in water at pH 6.5. A high fluorescence after the addition of liposomes or plasma membrane proves the interaction between these toxins and membranes. Fig. 3 shows the emission spectra of beticolin-2 in liposomes at different pH. Two forms were observed at an acidic pH. One with a fluorescence maximum at 465 and the other at 490 nm. The former corresponds to beticolin-2 dissolved in a non-polar medium. The second is probably a non-ionized beticolin-2 present in a polar phase because it has the same emission maximum as beticolin-2 in water at pH 3 [14]. This red shift could be amplified by pH-dependent specific interactions in the liposome membrane such as hydrogen bonds. As long as the pH increases, a dissociated form appears with the emission maximum at 513 nm. The excitation maximum shifted from 340 to 350 nm (not shown) which confirms that the beticolin form absorbing the light energy was dissociated.

The fluorescence of the ionized form of beticolin-2 in liposomes was 14-fold higher than that of beticolin-2 dissolved in 80% (v/v) dioxane. This difference might be due to interactions of dissociated group(s) with an electrophilic center in the biological membrane. The effect could be similar to that of magnesium ions forming a strongly fluorescent complex with the dianion (see below). The existence of aggregates or complexes on charged headgroups due to a high concentration of dyes in membrane was often reported. A detailed discussion was presented by Grzesiek and Dencher [17]. This hypothesis is supported by the fact that at pH 8, beticolin-2 did not fluoresce in laurylsulfate micelles but it fluoresced in micelles of tetradecyltrimethylammonium showing an emission maximum at 535 nm (unpublished results). Another explanation might be a localisation of some hydroxyl groups of beticolin-2 in the membrane-water interphase which is a heterogeneous polarity environment compared with a homogeneous solution.

When similar experiments were carried out with beticolin-1 or -2 and plasma membranes, the presence of several ionized forms was also observed (not shown). These results indicate that membrane-bound beticolins are situated in an environment allowing their dissociation.

3.2. The interaction of the complex beticolin-magnesium with membranes

The addition of magnesium ions to beticolin-2 in a hydro-organic solvent at neutral pH resulted in the

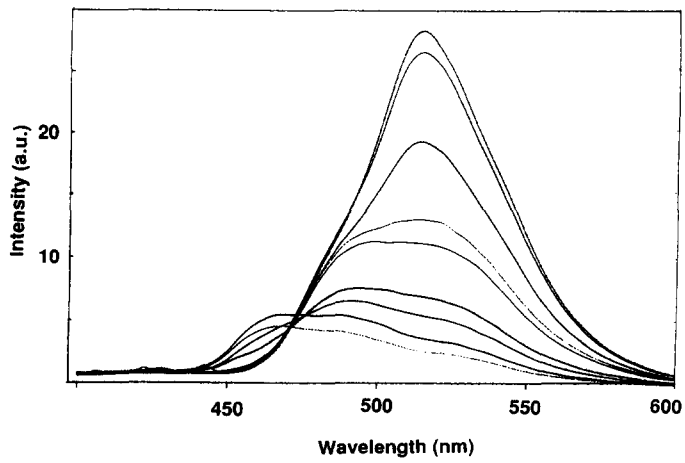


Fig. 3. Fluorescence emission spectra of beticolin-2 in the phospholipid membrane. 2.5 μM beticolin-2 and 0.4 mg phospholipids/ml in 20 mM Mes. Excitation wavelength was adjusted to 340 nm. From the bottom to the top: pH 3.0, pH 4.2, pH 5.4, pH 5.8, pH 6.4, pH 6.8, pH 7.2, pH 7.6, pH 8.2.

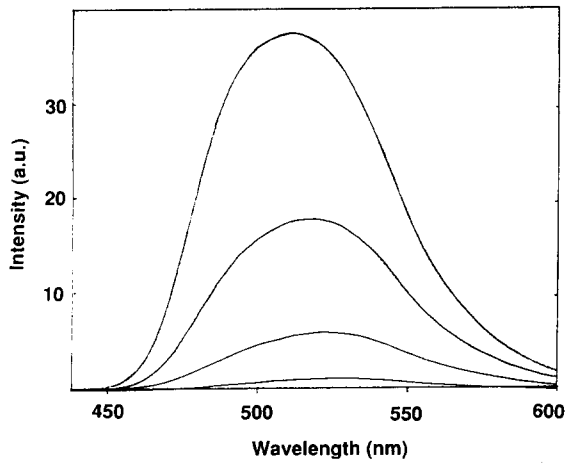


Fig. 4. Fluorescence emission spectra of the magnesium-beticolin-2 complex in a mixture (v/v) dioxane-50 mM Mes (pH 7.3). Excitation wavelength was adjusted to 350 nm. 5.5 μM beticolin-2 with 2 mM $MgCl_2$. From the top to the bottom: 80%, 60%, 40% and 20% dioxane.

formation of a fluorescent neutral complex [14]. Fig. 4 shows that the fluorescence of this complex was higher in less polar medium. Its fluorescence intensity in 80% (v/v) dioxane-Mes was about 9-fold higher than that of the dianion (Fig. 2). The fluorescence spectra of the mixture beticolin-2 with magnesium ions are presented in Fig. 5. At pH 3, no complex was formed and the emission spectrum was identical to that of the non-dissociated form. The addition of magnesium to liposomes shifted the equilibrium in favour of the form with the emission maximum at 513 nm. This corresponded to the emission maximum of both magnesium complex and of the dianion. The fluorescence intensities and maxima of dissociated and complex forms at

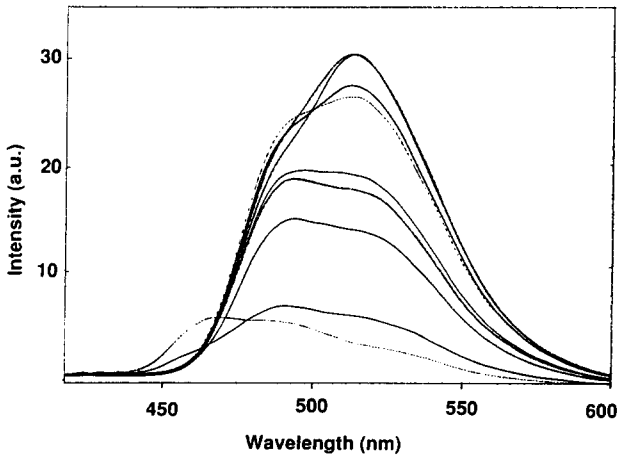


Fig. 5. Fluorescence emission spectra of beticolin-2 bound to the phospholipid membrane in the presence of magnesium ions. 0.1 mg phospholipids/ml, 2.5 μM beticolin-2 and 2 mM $MgCl_2$ in 20 mM Mes. Excitation wavelength was adjusted to 345 nm. From the bottom to the top: pH 3.0, pH 4.2, pH 5, pH 5.3, pH 5.8, pH 6.2, pH 6.4, pH 7.1, pH 7.5

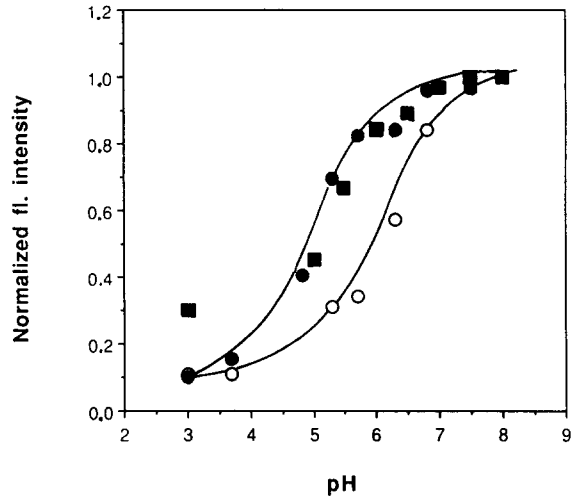


Fig. 6. Comparison of the effect of pH on the fluorescence of beticolin-2 at 515 nm on liposomes and in 50% (v/v) dioxane-Mes. The fluorescence intensities on liposomes were read from Fig. 3, 4. The maximal intensities were normalized to 1.0. ○, 2.5 μM beticolin-2 on liposomes; ●, 2.5 μM beticolin-2 with magnesium on liposomes; ■, 5 μM beticolin-2 with 2 mM $MgCl_2$ in 50% (v/v) solution dioxane-50 mM Mes.

high pH were the same (Figs. 3 and 5). The exact identity of the form which fluoresces at 513 nm remains unclear since this result did not allow us to discriminate between the magnesium complex and the dianion.

We reported previously [14] that the fluorescence at 513 nm in 50% (v/v) dioxane-water was proportional to the concentration of the neutral magnesium complex of beticolin-1 and -2. The fluorometric pH titration curve of beticolin-2 with magnesium in liposomes corresponded to the same curve obtained with the magnesium complex in 50% (v/v) dioxane-50 mM Mes. The curve of beticolin-2 in liposomes without magnesium shifted to a higher pH (Fig. 6). Magnesium is believed to screen the surface charges and shifts of pK_a values of acidic bound dyes are usually observed after the addition of magnesium to membranes [18]. Although the coincidence of the curves of beticolin-2 with magnesium in liposomes and in the dioxane solution points to the presence of the complex the effect of magnesium on the binding due to a screening of the surface charges is also possible.

The neutral complex of beticolin in 20 mM Mes in excess of magnesium was extracted with n-octanol. The concentration of beticolin-2 in aqueous phase after the extraction was negligible. In the octanol phase, the magnesium concentration as well as the fluorescence of the complex at 510 nm were measured simultaneously (Fig. 7). The figure shows that no magnesium was extracted from the aqueous phase by the octanol up to pH 4.0. The magnesium concentration and the concentration of the complex increased with rising pH and

both curves corresponded well. The ratio of the concentration of beticolin-2 to magnesium was about 1 at pH 8.0. This is in agreement with a dimeric structure $Mg_2H_2B_2$ isolated by Jalal et al. [12].

These results indicate that the existence of the uncharged complex in a hydrophobic region of the membrane is not excluded and that its concentration depends on pH of the aqueous phase.

3.3. Distribution of beticolins across the membrane

Beticolin-2 (1–10 μM) does not exert an uncoupling effect and does not disturb the diffusion potential created by valinomycin and potassium in liposomes (unpublished results). It proves that the dissociated form cannot freely penetrate the membrane. However, some experiments indicate that beticolins can react to ΔpH changes across the membrane and not only to pH near the membrane surface (Fig. 6). We prepared liposomes and imposed a pH gradient. In order to eliminate the direct effect of a water phase, the pH range was taken from 6.6 to 8.2. In this range the fluorescence of beticolin-2 did not change much (see the curve in Fig. 6). The fluorescence of beticolin-2 neither depended on the pH of the inner phase nor on the pH of the outer phase. However, it depended on ΔpH (Fig. 8). The highest intensity was observed with

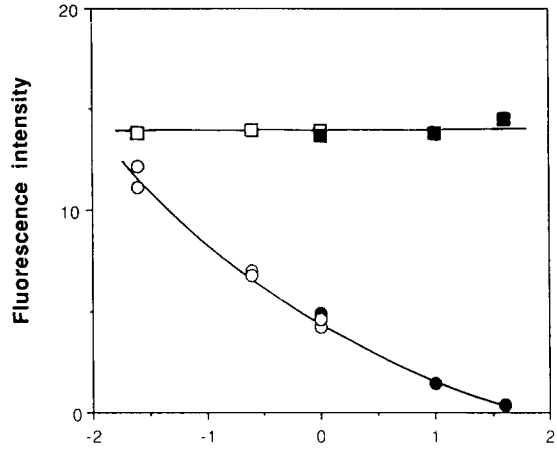


Fig. 8. Fluorescence response of beticolin-2 on ΔpH across the liposome membrane. The liposomes (0.1 mg/ml) prepared in 20 mM Mes-NaOH (pH 6.2 and 7.2) or in Tes-NaOH (pH 8.2) were added to the same buffer of different pH. 0.1 mM EDTA was added to all solutions. Thereafter, beticolin-2 (1.6 μM) was added and the fluorescence was read after 10 s. ΔpH is expressed as the difference of the outer and inner pH. The outside buffer concentrations were: ○, 20 mM Mes-NaOH (pH 6.6); ●, 20 mM Tes-NaOH (pH 8.2); □, 20 mM Mes-NaOH (pH 6.6) with 1 mM $MgCl_2$; ■, 20 mM Tes-NaOH (pH 8.2) with 1 mM $MgCl_2$.

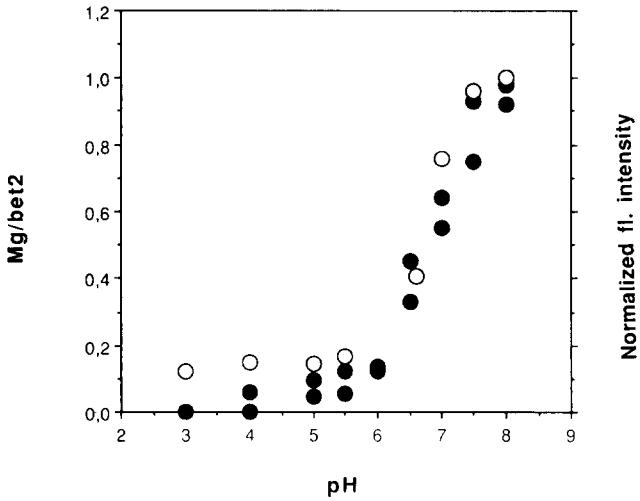


Fig. 7. Ratio of molar concentration of magnesium/beticolin-2 extracted from 20 mM Mes by n-octanol at different pH. 3 ml 20 mM Mes (pH 3–8) were added to 3 ml 0.1–0.5 mM beticolin-2 in n-octanol. After the addition of 10 mM $MgSO_4$, test tubes were intensively shaken under nitrogen for 1 min. The octanol phase was collected, centrifuged to eliminate traces of water and the fluorescence of the complex was measured at 510 nm. The concentration of beticolin-2 in water was neglected. The beticolin-magnesium complex in the octanol phase was split by the addition of 3 ml 20 mM Mes (pH 2.5). Next, the magnesium concentration reextracted with aqueous phase was determined by atomic absorption. The control sample contained 20 mM Mes (pH 2.5) at the beginning of the experiment. ●, ratio magnesium/beticolin; ○, normalized fluorescence intensity.

the liposomes which were alkaline inside and acidic outside. The presence of magnesium in the outer space abolished completely this effect whatever the value or the sign of ΔpH. A similar experiment shows that the alkalinisation of the formely acidic internal space of liposomes by addition of ionophores caused a redistribution of the membrane bound beticolin-2 and an increase in its fluorescence (Fig. 9a). The addition of magnesium chloride to liposomes gave rise to the strongly fluorescent complex. According to Fig. 8, the alkalinisation of the inner space did not induce any redistribution of the beticolin complex in the membrane (Fig. 9b).

The fluorescence reaction of beticolin-2 was compared with that of an aminoacridine derivative used as a fluorescent probe for the measurement of ΔpH [19,20]. The addition of FCCP and valinomycin to liposomes with an acidic internal space resulted in an increase in the aminoacridine fluorescence (Fig. 9c). The addition of magnesium ions had no effect on the fluorescence response of the aminoacridine dye (trace d). The nature of the fluorescence reaction of beticolin-2 to ΔpH is not clear. It could be a result of the transport of the uncharged form of beticolin-2 across the membrane, a mechanism stated for the aminoacridines [19]. Such a transport was also suggested for lipophilic tetracyclines [21] which are passively distributed, as weak acids, across the bacterial wall according to the pH gradient. Our results indicate that

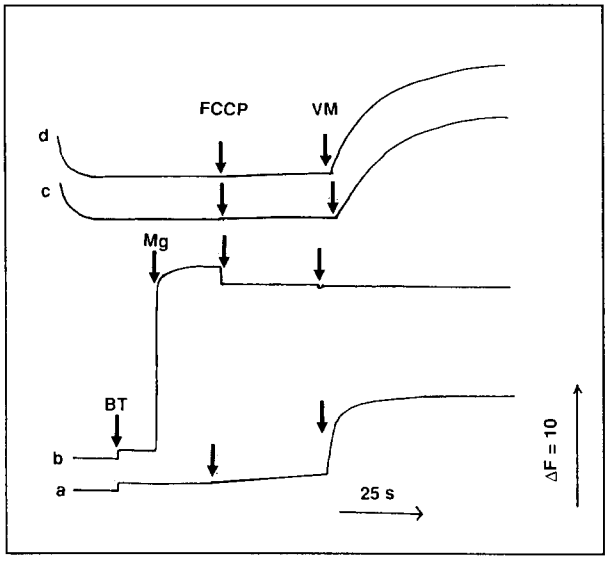


Fig. 9. The fluorescence response of beticolin-2 and of aminoacridine dye on ΔpH changes in liposomes stimulated by ionophores. Liposomes (0.1 mg/ml) prepared in 20 mM Mes-NaOH (pH 6.5) with 0.1 mM EDTA were added to the solution of 20 mM Tes-NaOH (pH 8.0) with 0.1 mM EDTA and 0.5 mM K_2SO_4. The alkalinisation of the internal space was induced by 3 μM FCCP followed by 0.7 μM valinomycin (VM). The measurement of beticolin-2 (1.6 μM; BT) fluorescence was carried out at the excitation wavelength adjusted to 340 nm, emission wavelength to 513 nm without magnesium (trace a) and with 2 mM $MgCl_2$ (trace b); the measurements of 9-amino-6-chloro-2-methoxyacridine (1 μM) fluorescence at the excitation wavelength adjusted to 430 nm, emission wavelength to 500 nm without magnesium (trace c) and with 2 mM $MgCl_2$ (trace d).

only the bound beticolins fluoresce at a physiological pH and that these phenomena take place at the membrane surface. Grzesiek and Dencher [17] showed that the quenching of the aminoacridines in the membrane is proportional to the number of dimer-excimer complexes, which seems to be the photophysical mechanism of the fluorescence decrease. The explanation of the nature of the effect of the pH gradient on the aminoacridine fluorescence is less clear. These molecules might penetrate into the internal space where they form complexes with the negative lipid headgroups [17]. On the other hand, evidence was put forward by Kraayenhof et al. [22] that the penetration is not required for the quenching and the molecules bind outside. Regardless of the mechanism of the fluorescence response of beticolins to the ΔpH, the presence of magnesium removes beticolins from these equilibria by a strong binding of the compounds to the membrane.

3.4. Binding parameters of beticolins on phospholipids and plasma membranes

The binding parameters of beticolins, i.e., the partition coefficient in the absence and the presence of magnesium as well as the fluorescence were determined by the fluorometric titration of beticolins with phospholipids or plasma membranes. As the hydrophobicity of beticolins is high they were added to the fluorometric cuvette after the membranes. The fluorescence intensity was recorded after the stabilisation. The binding parameters of beticolins in the plasma membrane in the absence of magnesium could not be determined because the double reciproçal plot was not linear, probably due to the necessity of the long incubation or to the presence of several binding sites. The results are shown in Table 1. The partition coefficient of beticolin-1 in liposomes was 3-times that of beticolin-2. The addition of magnesium to liposomes markedly increased the affinity of beticolin-1 and -2 so that their partition coefficients increased 40- and 20-times, respectively. The addition of 100 mM NaCl which neutralizes the negative surface potential and usually influences the surface binding of charged compounds [23] had only a small effect on the partition coefficient of beticolins. The distribution coefficient of the magnesium complex of beticolin-2 in the octanol/50 mM Mes (pH 6.5) mixture was lower than that of free beticolin (unpublished results). This suggests that the interaction is not a simple extraction of the complex into the membrane and that the complex should be situated in the headgroup region and not in the nonpolar region of the membrane. The increase in the partition coefficient in liposomes is due to a specific interaction between beticolin, magnesium ions and membrane surface rather than to the supposed effect of magnesium ions on the surface potential.

The inhibitory effects of CBT on ATPase are stronger at high pH [6]. Table 2 shows the effect of the pH on the binding parameters of the magnesium-beticolin-2 complex in phospholipids and plasma membranes. It can be observed that the fluorescence intensity of the complex on liposomes and on plasma mem-

Table 1
Partition coefficients and fluorescence of beticolins bound to liposomes

Compound	F_b (a.u./μM)	P (ml/mg)
Beticolin-1	7.4	10.1
+ $MgCl_2$	6.9	419.0
Beticolin-2	8.5	3.8
+ NaCl	8.1	6.4
+ $MgCl_2$	10.5	85.0

1–2 μM beticolin in 20 mM Mes (pH 6.5) and 0.1 mM EDTA were titrated with liposomes (0.002–0.4 mg/ml). The fluorescence was followed with an excitation wavelength adjusted at 340 nm and emission wavelength at 513 nm. The concentration of $MgCl_2$ and NaCl was 2 mM and 100 mM, respectively. The fluorescence of the bound derivative (F_b) is expressed in arbitrary units/μM and the partition coefficient on liposomes (P) in ml/mg phospholipids. The correlation coefficients of the straight lines were 0.96–0.99.

Table 2
The effect of pH on the interaction of magnesium complex of beticolin-2 with liposomes and plasma membranes

pH	F_b (a.u./μM)		P (ml/mg)	
	Lp	Pm	Lp	Pm
5.0	–	7.9	–	0.12
5.5	8.0	7.5	89	0.17
6.0	10.0	10.5	78	0.16
6.5	10.5	13.5	85	0.14
7.0	12.3	13.6	87	0.13
7.5	12.0	–	78	–

1.3–1.7 μM beticolin-2 in 20 mM Mes (pH 6.5) with 2 mM $MgCl_2$ was titrated with liposomes (0.002–0.02 mg/ml, Lp) or plasma membrane (0.002–0.01 mg protein/ml, Pm). The partition coefficient P is expressed in ml/mg phospholipids or in ml/mg protein. Other conditions are given in Table 1. The correlation coeffficients of the straight lines were 0.95–0.98.

brane was the same. The changes of pH did not bring about any marked changes of the partition coefficient of the complex. The increase in the fluorescence of the bound compounds was the only effect of pH, which agrees with the results of Fig. 5 showing that the concentration of the complexes on liposomes increases at high pH. It confirms the hypothesis that the stronger inhibition of plasma membrane ATPase by beticolins at high pH could be due to the increase in the concentration of an inhibitory form and not to higher 'partition coefficient' of the inhibitor in the membrane.

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