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Effect of the *O*-methylation of tyrosine on the pore-forming properties of iturins

R. Maget-Dana [a], M. Ptak [a], F. Peypoux [b] and G. Michel [b]

[a] *Centre de Biophysique Moléculaire, C.N.R.S. and Université d'Orléans, 1A, avenue de la Recherche Scientifique, 45071 Orléans Cedex 2 (France) and* [b] *Laboratoire de Biochimie Microbienne, Université de Lyon I, 43, boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex (France)*

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A comparison has been made between the pore-forming properties of the antibiotic lipopeptide iturin A and a derivative methylated on the tyrosine residue which shows a restricted biological activity. It is shown that this derivative increases the ion permeability of planar lipid membranes as does iturin A. Nevertheless, the global conductance of the doped membrane is very much lower at the same lipopeptide/phospholipid ratio and the ion selectivity is inverted ($P_K/P_{Cl} = 6$ instead of 0.6 with iturin A). The characteristics of the induced conducting pores are also rather different. This suggests an important role of the D-Tyr^2 residue, present in all the compounds of the iturin family, both in the biological and in the pore-forming properties of iturin A.

Introduction

The antibiotic activity of the bacterial lipopeptides forming the iturin family is thought to be related to their interaction with membranes [1]. This activity depends on the primary structure of the peptide cycle [2–4], which is characterized by an invariable chiral sequence LDDLLDL and a D-tyrosine residue in position 2 [5], as well as on the membrane composition. We have recently demonstrated that these lipopeptides are able to modify the permeability of planar membranes by inducing ion conducting pores [6,7]. This capability depends also on both the lipid composition of the membrane and the structure of the lipopeptides. There is then a parallel between the antibiotic and the pore-forming properties but their relationship remains to be definitively proved. As far as the structure of the peptide moiety is concerned one can attribute a special role to the D-tyrosine residue which is present in position 2 in all iturins. Its *O*-methylation in iturin A and other related compounds dramatically decreases the antibiotic activity [2,3]. In the present work we report the effect of such substitution on the pore-forming capacity of iturin A in planar membranes.

Correspondence: R. Maget-Dana, Centre de Biophysique Moléculaire, 1A, avenue de la Recherche Scientifique, 45071 Orléans Cedex 2, France.

Material and Methods

Iturin A was prepared as described previously [5] and the *O*-methyl derivative according to Ref. 2. Egg phosphatidylcholine was prepared in our laboratory by M. Charlier according to Ref. 8. Cholesterol from Prolabo was recrystallized twice from ethanol. KCl, analytical grade was from Merck. All methods concerning planar lipid membranes are described in Ref. 6. Briefly, planar membranes were formed from lipid vesicle suspensions [9]. The lipopeptide was added directly to the lipid vesicle suspension in order to obtain a lipopeptide/lipid molar ratio ranging between 10^{-10} and 10^{-5} for the steady-state conductance

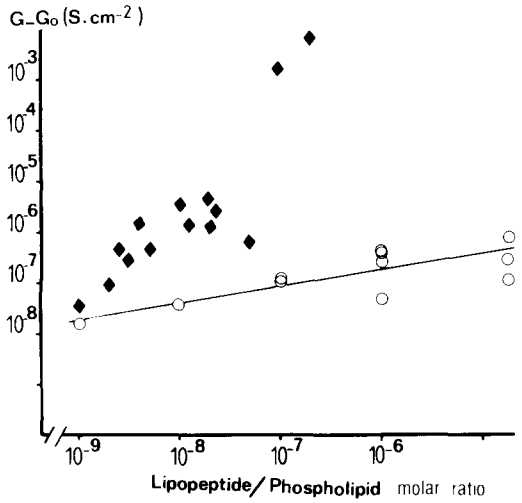

Fig. 1. Macroscopic conductance of the membrane versus lipopeptide concentration. The planar membrane was formed from egg phosphatidylcholine vesicles and separated two symmetrical 0.1 M KCl solutions. The lipopeptide was added to the vesicle suspension at least 60 min before the formation of the planar membrane. Each point represents a different membrane. The plot is semi-logarithmic. The macroscopic conductance of the membrane in the absence of lipopeptide was $G_0 = 10^{-8}$ S·cm^{-2}. ◆, iturin A; ○, *O*-methyltyrosine-iturin A.

experiments and below 10^{-10} for the observation of discrete pore events. The electrical measurements were made via a pair of Ag/AgCl electrodes. The determinations of ion selectivity were done from the measurements of the 'zero-current potential' when a concentration gradient of KCl was established across the membrane.

Results

Steady-state conductance

In the absence of lipopeptide, the steady-state conductance G_0 of the egg phosphatidylcholine membrane was around 10^{-8} S·cm^{-2}. By adding iturin A to the egg phosphatidylcholine vesicles before the formation of the planar membrane the conductance observed could then be increased up to 10^{-3} S·cm^{-2} at a lipopeptide/phospholipid molar ratio of 10^{-7}. Above this molar ratio the planar membrane became unstable and broke. When the planar membrane was doped with the methylated derivative the increase in the macroscopic conductance was very much lower: at a lipopeptide/phospholipid molar ratio of 10^{-5} it was only 10^{-6} S·cm^{-2}, i.e., 100 times that the undoped egg phosphatidylcholine membrane (see Fig. 1).

Conducting pores

At a lipopeptide/phospholipid molar ratio lower than 10^{-10}, one can observe membrane current fluctuations that correspond to opening and closing of conducting pores. We have compared the characteristics of the current fluctuations induced by iturin A and the methylated derivative. For the two compounds the conduc-

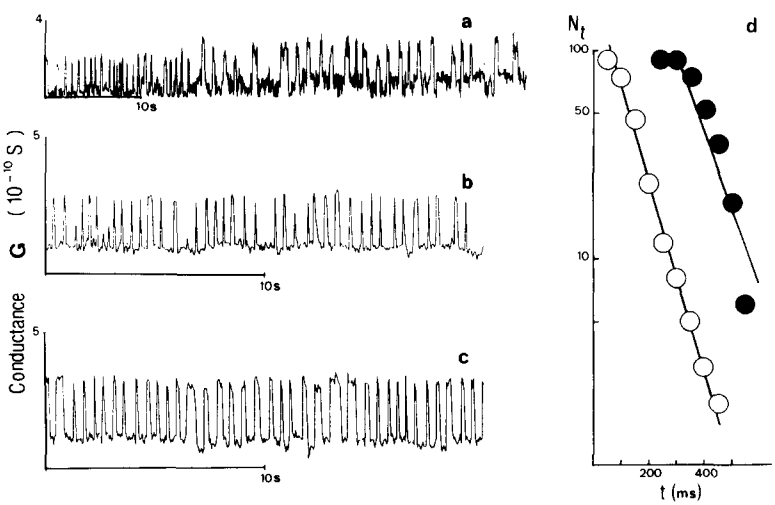

Fig. 2. Membrane current fluctuations induced by *O*-methyltyrosine iturin A in planar membrane. The planar membrane was made from egg phosphatidylcholine vesicles in KCl 0.5 M. Time after formation of the membrane: (a) 2 min, (b) 10 min, (c) 20 min. (d) Distribution of the dwell-times in open and closed state 20 min after the membrane was formed. N_t is the number of individual dwell-times longer than t. The plot is semi-logarithmic. ○, dwell-time in the open state; ●, dwell-time in the closed state.

tance value of the induced pores was not dependent on the voltage applied and seemed to vary at random from one pore to another. Furthermore in all cases we could observe an increase with time both in the base conductance of the membrane and in the conductance of the single event. This last property, already reported for iturin A [6,7], is illustrated in Fig. 2 for the methylated derivative. In this figure we can notice the establishment of a first step in the increase of the base conductance of the membrane (Fig. 2a) as well as a continuous increase in the conductance of the pore. Thus it is not possible to define a conductance value for the pore induced by either lipopeptide. We can notice also that the dwell-time in the closed state varied only between 300 and 500 ns (Fig. 2d). As a result the opening events appeared rather regularly spaced (Figs. 2b and 2c). Such a special behaviour, already observed with iturin A [6] is not still elucidated.

We have studied the kinetics of the opening and closing of the pores. The mean dwell time in the open state (which was around 1000 ms for the pores induced by iturin A [6]) was at most 200 ms in the case of the methylated derivative (Fig. 2d). As shown in Fig. 3, the presence of cholesterol in the membrane bilayer changed the opening and closing rates of the induced pores. In the case of iturin A, the kinetics were then very slow and the pores could stay in the open or closed state for more than 1 min. The conducting pores induced by the methylated derivative were also sensitive to cholesterol. The mean dwell-time in the open state was then around 300 ms with some long lasting events of about 10 s. Furthermore we could observe two levels in the pore conductance which were not integral multiples of each other: the second level value was roughly 1.5-times that of the first one.

Ion selectivity

The ion selectivity given by the permeability ratio P_K/P_{Cl} is calculated according to the Goldman-Hodgkin-Katz equation [10]

$$V_m = \psi_1 - \psi_2 = \frac{RT}{F} \ln \frac{P_K a_2 + P_{Cl} a_1}{P_K a_1 + P_{Cl} a_2}$$

(V_m, membrane potential; a, KCl activity on each side 1 and 2 of the membrane).

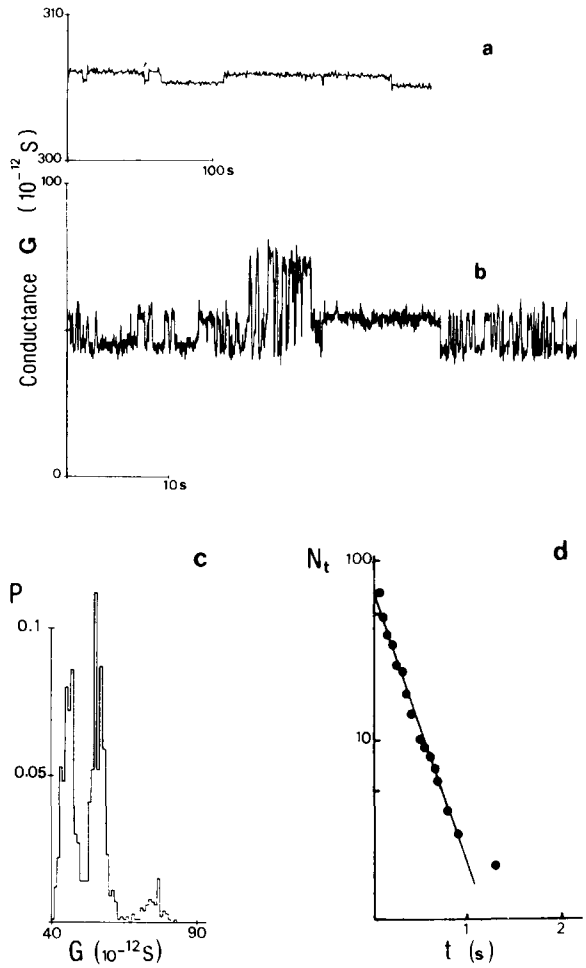

Fig. 3. Membrane current fluctuations induced by lipopeptides in cholesterol-containing membrane. The planar membrane was made from egg phosphatidylcholine/cholesterol (4:1) vesicles in 0.5 M KCl. (a) Iturin A. (b) *O*-Methyltyrosine-iturin A. Note the difference in the time scale. (c) Conductance histogram for *O*-methyltyrosine-iturin A showing the occurrence of two conductance levels which were not integral multiples of each other. (d) Distribution of the dwell-time in open state for *O*-methyltyrosine-iturin A.

The membrane potential V_nm was deduced from the measure of the 'zero-current potential' V_z by the relation $V_z = V_m + (RT/F) \ln(a_1/a_2)$ that takes into account the potential of the Ag/AgCl electrodes.

Fig. 4 shows the variations of the membrane potential V_m versus the activity ratio a_1/a_2 of KCl. We can see that the methylation of the

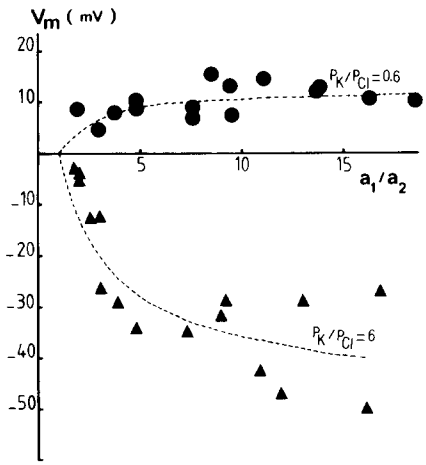

Fig. 4. Ion selectivity induced by lipopeptides in egg phosphatidylcholine membrane. The membrane potential V_m is plotted versus the ratio a_1/a_2 of the KCl activities on both sides of the membrane. The dashed lines are drawn according to the Goldman-Hodgkin-Katz equation [10] with $P_K/P_{Cl} = 0.6$ for iturin A (●) and with $P_K/P_{Cl} = 6$ for *O*-methyltyrosine-iturin A (▲).

tyrosyl group of iturin A reversed the ion selectivity of the doped membrane which became cation selective ($P_K/P_{Cl} \approx 6$ instead of 0.6 for iturin A).

Discussion

The *O*-methylation of the D-tyrosine residue has an effect on the mechanism by which iturin A modifies the permeability of planar membranes: the value of the global conductance, the dynamics of the pores and the ion selectivity are changed as compared with the unmodified compound. Such a substitution could be considered a priori as a minor change of the peptide moiety. The tyrosyl group lies on the surface of the molecule and is not involved in any intramolecular stabilization process [11]. In iturin A, the tyrosyl group is free to form a hydrogen bond through its OH group. The methylation suppresses this possibility and increases the local hydrophobicity. Nevertheless its important consequence on the activity of iturin A, already mentioned in the course of the biological studies [2,3], emphasizes the role of the D-tyrosine residue.

Two kinds of mechanisms could be considered for the pore-forming activity of lipopeptides. As a molecule of iturin A cannot form a channel by itself, one could assume that conducting pores are formed by transient structures corresponding to an association of several molecules of iturin A or to mixed iturin A-lipid structures.

We have also to consider that conducting defects normally occur in the lipid membranes [12]. The presence of lipopeptide aggregates within the lipid bilayer can amplify these defects by increasing the mobility of the surrounding phospholipid molecules [13]. The importance of the lipid composition as evidenced by the effect of cholesterol on the dynamics of the pores is rather in favor of the second assumption whereas the sharp dependence of the activity on the structure of the peptide supports more the first one. The *O*-methylation of the tyrosyl side chain can modify the mode of self association of iturin A as well as its interaction with lipids. A more decisive argument is the change in the ion selectivity of the doped membrane which suggests that iturin A is directly involved in the ion conduction. The small anion selectivity induced by iturin A becomes a pronounced cation selectivity with the *O*-methylated derivative. Concerning the conduction process one must note that in addition to current fluctuations there is a continuous increase of the base conductance of the membrane which could be due to a direct transport of ions by the lipopeptides. Very recently, Marion et al. have proved that iturin A interacts with ions specially in the region of the D-tyrosine residue (D. Marion et al., to be published).

All these features show that the region of the D-tyrosine residue plays a decisive role in the interaction of iturin A with the bilayer and possibly with ions.

The study presented here shows that a minor modification in the peptidic sequence, which considerably decreases the biological activity, modulates too the pore forming properties of iturin A. This result is relevant to the general problem of the relationship between the antibiotic power of many compounds and their ability to induce conducting pores in the membranes [14]. To establish this relationship it will be necessary to know actually the pore induction process. We have at our disposal a family of compounds possessing variable biological activities correlated with a change in the peptidic sequence. We have now to study how

these changes influence the self-association process and the interaction of the lipopeptide with lipids and with ions in order to understand the whole phenomenon of the induction of conducting pores in membranes.

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