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Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A

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Abstract

The bacterial lipopeptide iturin A is able to cause hemolysis of human erythrocytes in a dose-dependent manner. Hemolysis takes place at iturin concentrations below its critical micellar concentration. Relative kinetics determinations clearly show that K^+ leakage occurs prior to hemoglobin release. Furthermore, hemolysis can be prevented by addition to the outer solution of osmotic protectants of appropriate size. Altogether these results indicate that iturin A-induced hemolysis follows a colloid-osmotic mechanism, with the formation of a membrane pore of average diameter 32 Å. Iturin A is capable of inducing leakage of an aqueous fluorescent probe trapped in human erythrocyte ghosts, but not in large unilamellar liposomes made of various lipid compositions. The different permeabilizing effects of iturin A on model and biological membranes are discussed on the light of the presented results.

Keywords: Iturin; Lipopeptides; Biosurfactants; Liposomes; Hemolysis

1. Introduction

Biosurfactants, as compared with synthetic compounds, are in general more selective, effective, stable and, therefore, environmentally friendly, presenting a number of very interesting and promising technical applications [1,2]. Many microorganisms, particularly bacteria, release biosurfactants to their growth medium. The chemical nature of these compounds is rather heterogeneous, including glycolipids, lipoproteins, heteropolysaccharides and lipopeptides [3].

Iturin A, a lipopeptide which was originally isolated from the culture medium of a strain of *Bacillus subtilis* [4], behaves as a notable biosurfactant, and therefore, has been devoted to a great deal of attention up to date [5]. The critical micellar concentration (CMC) of iturin A is ca. 25 μM, very close to the minimal inhibitory concentration against the growth of *Saccharomyces cerevisiae*, which is 35 μM [6]. The structure of iturin A consists of two major parts: a peptide ring composed of seven amino acid residues and an 11–12 carbons hydrophobic tail (Fig. 1). This structure clearly indicates a marked amphiphilic character for this compound, thus, pointing to the cellular membranes as the most probable site of its action. In fact, iturin A exhibits strong antifungal [7] and hemolytic [8] activities, which have been ultimately explained as the result of the interaction of the lipopeptide with the membrane of its target cells.

In addition, it has been shown that iturin A forms ion-conducting pores in planar lipid bilayers [9,10], which has been explained as the result of the formation of aggregated structures in the lipid membrane [11,12]. In fact, iturin A has been shown to be able to perturb the structure of membranes by modification of the curvature of phospholipid vesicles [13]. The hemolytic activity of iturin A has been reported in a previous work [14], however, it has not been established any possible relationship between the ion-forming capacity of iturin A and its hemolytic activity. In addition, the mechanism of the hemolytic activity of iturin and the process of iturin A-induced membrane permeabili-

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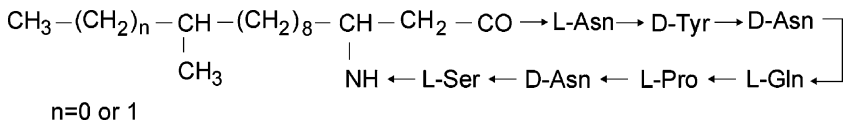

Fig. 1. Primary structure of iturin A.

zation need to be further characterized. In this paper, we present experimental evidence to explain the mechanism of iturin A-induced hemolysis, together with a thorough characterization of the process.

2. Materials and methods

2.1. Materials

Iturin A was prepared and purified from *Bacillus subtilis* as previously described [15]. 5(6)-Carboxyfluorescein (CF) was purchased from Sigma-Aldrich (Madrid, Spain). Natural and synthetic phospholipids were from Avanti Polar Lipids Inc. (Birmingham, AL). All the other reagents were of the highest purity available. Water was twice-distilled in an all-glass apparatus and deionized using a Milli-Q equipment from Millipore (Bedford, MA). Stock solutions of the various phospholipids were prepared in chloroform/methanol (1:1) and stored at −20 °C. Iturin A was added to the aqueous buffers from a 5-mM stock solution prepared in DMSO.

2.2. Erythrocytes

Human erythrocytes were freshly prepared right before the experiments from outdated red blood cell concentrates obtained from a local blood bank. Cells were washed twice with buffer (150 mM NaCl, 5 mM HEPES, pH 7.4), and finally suspended in this same buffer at a hematocrit of 24%. All the operations were carried out at 4 °C.

2.3. K^+ leakage and hemolysis

The leakage of K^+ from erythrocytes was measured using a K^+-selective electrode (Jenway, UK) as follows. Erythrocytes were suspended in 4 ml of 150 mM NaCl, 5 mM HEPES, pH 7.4, at a hematocrit of 0.36%. Iturin A was added from a 5-mM stock solution in DMSO, and the potassium efflux was continuously monitored using a recorder. The total amount of K^+ was determined by disrupting the cells with sodium cholate. The solutions were continuously stirred using a magnetic device. Measurements were done in a jacketed vessel and temperature was kept constant using a circulating water bath.

Upon incubation of red blood cells with iturin A under different conditions as indicated, hemolysis was determined by measuring the absorbance of released hemoglobin at 540 nm after pelleting the membranes by centrifugation for 2 min in a bench microfuge. The total amount of hemoglobin was established by lysing the erythrocytes with water.

2.4. Osmotic protection experiments

Erythrocytes were suspended (0.36% hematocrit) in a buffer containing 135 mM NaCl, 5 mM HEPES, pH 7.4 and 30 mM of one of the following substances: sucrose and polyethylene glycols 400, 600, 1000, 4000, 6000 and 10000. Iturin A was added to the different final concentrations indicated in each experiment from a 5-mM stock solution in DMSO, and hemolysis was determined after incubation for 30 min at 25 °C. The following molecular diameters were used [16]: sucrose, 9.8 Å; PEG 400, 11.2 Å; PEG 600, 13.8 Å; PEG 1000, 17.8 Å; PEG 4000, 32 Å, PEG 6000, 54 Å and PEG 10000, 72 Å.

2.5. Vesicle preparation

Vesicles to be used in the CF leakage assay were prepared by mixing the appropriate amount of lipids in chloroform/methanol 2:1. The solvent was gently evaporated under a stream of dry N_2 to obtain a thin film at the bottom of a small thick-walled glass tube. Last traces of solvent were removed by a further 2 h dessication under high vacuum. To the dry samples 1 ml of a medium containing 50 mM CF, 5 mM HEPES pH 7.4 was added and multilamellar vesicles were formed by vortexing the mixture. Large unilamellar vesicles (LUV) were prepared by repeated extrusion of the multilamellar vesicles through 0.1 μm polycarbonate filters (Nuclepore, Pleasanton, CA, USA). Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-50, using 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES pH 7.4 as elution buffer.

Phospholipid phosphorous was determined according to the method of Böttcher [17].

2.6. Leakage of vesicles contents

Release of CF was measured in the buffer indicated above in a Shimadzu RF-540 spectrofluorometer. Excitation and emission wavelengths were 430 and 520 nm, respectively. For calibration of the fluorescence scale, maximum release was induced by lysing the vesicles with 1% (v/v) Triton X-100. Iturin A was added to vesicles suspensions in buffer from a stock solution in DMSO. The volume of DMSO added was always less than 10% of the total buffer volume and, in any case, it was checked that DMSO by itself did not induce any leakage. Furthermore, in some experiments, the concentration of peptide was changed

maintaining a fixed volume of DMSO and it was observed that this solvent did not affect leakage (not shown). All measurements were done at 25 °C.

3. Results and discussion

3.1. Disruption of erythrocyte membranes by iturin A

The capacity of iturin A to cause hemolysis of human erythrocytes has been reported before [8,14,18]. These authors have informed on the ability of the lipopeptide to lyse erythrocytes in a dose-dependent manner, as well as on the affinity of iturin A for cholesterol-containing membranes. However, up to our knowledge, the mechanism of iturin A-induced hemolysis was not unveiled, nor any relationship to its pore-forming activity was established. In this work, we have carried out a detailed study of the hemolytic activity of iturin A in order to obtain conclusions on the type of molecular mechanism which is operating.

Fig. 2 shows the dependence of iturin A-induced hemolysis on the concentration of the lipopeptide at two different temperatures. The results obtained were essentially the same both at 25 and 37 °C and therefore, for the shake of simplicity, the rest of the work was carried out at 25 °C. It can be observed that the threshold concentration of iturin A causing the release of hemoglobin was around 10 μM, and at 25 μM and above hemolysis was complete. All the iturin A concentrations studied were essentially below its CMC, which has been reported to be around 25 μM [5], indicating that the action of the lipopeptide on the erythrocyte membrane is not due to membrane disruption or solubilization.

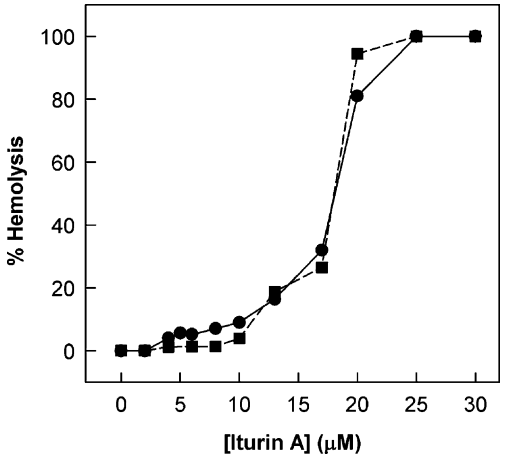

Fig. 2. Dependence of the relative hemolysis of human erythrocytes on the concentration of iturin A. Iturin A was added to a human RBC suspension to the desired final concentrations from a stock solution in DMSO. Incubations were carried out for 30 min at 25 °C (squares) and 37 °C (circles) and hemolysis was determined as explained in the text. Data correspond to the mean of three different experiments. Error bars are shown when bigger than the symbols.

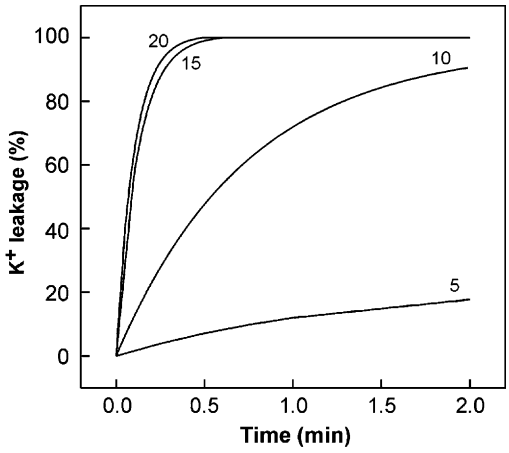

Fig. 3. Time dependence of iturin A-induced K^+ leakage from human erythrocytes. Iturin A was added to a human RBC suspension to the desired final concentrations (indicated on the curves) from a stock solution in DMSO, and K^+ leakage was continuously monitored using a K^+-selective electrode. Incubations were carried out at 25 °C under continuous stirring.

Upon interaction with red cells iturin A also induced the leakage of K^+ ions (Fig. 3) in a concentration-dependent manner. From the curves shown in Fig. 3, initial rates and extents were obtained and the results are shown in Fig. 4. It can be seen that increasing the concentration of iturin A increased both the initial rate (panel A) and the extent (panel B) of K^+ leakage. It is interesting to note that, at difference with the hemolysis shown in Fig. 2, concentrations of iturin A as low as 5 μM already induced an efflux of K^+ from erythrocytes, whereas hemoglobin was not yet released. Furthermore, a comparison of the curves shown in Figs. 2 and 4 clearly shows that concentrations of iturin A around 10 μM, which induced more than 80% of K^+ leakage after 1 min (Fig. 4, panel B) were essentially no hemolytical (Fig. 2). All these data suggest that K^+ leakage occurs prior to hemolysis. Nonetheless, this point was evaluated more in detail as described below.

3.2. Relative kinetics of K^+ leakage and hemolysis

In order to establish the mechanism of iturin A-induced hemolysis, the relative kinetics of K^+ leakage and hemolysis of human erythrocytes were measured and compared (Fig. 5). Iturin A caused a rapid leakage of K^+, which was essentially complete within 1 min at this iturin concentration (Fig. 5, inset), whereas hemolysis occurred gradually, suggesting that the lipopeptide produced a membrane lesion of a rather small size, hemolysis being the consequence of a colloid-osmotic process [19]. This result is in contrast with the conclusion presented before by Latoud and co-workers [14]. These authors concluded that iturin caused the simultaneous release of hemoglobin and K^+, however no kinetic evidence was presented. Fig. 5 clearly shows the relative kinetics of the two processes, and

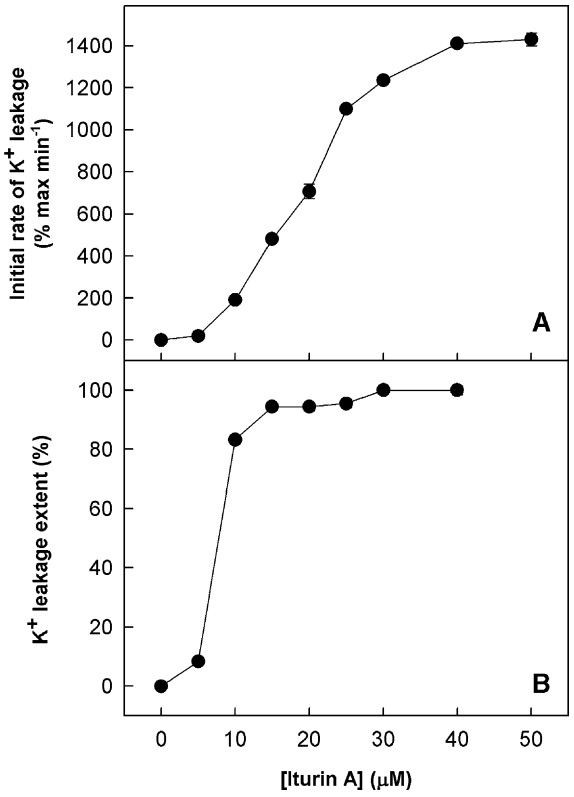

Fig. 4. The effect of increasing concentrations of iturin A on K^+ leakage from human RBC. Iturin A was added to a human RBC suspension to the desired final concentrations from a stock solution in DMSO, and K^+ leakage was continuously monitored using a K^+-selective electrode. Initial rates and extents after 1 min were determined from the curves after lysing the cells with 0.5% sodium cholate. Measurements were done at 25 °C. Data correspond to the mean of three different experiments. Error bars are shown when bigger than the symbols.

this let us to establish the mechanism of iturin A-induced hemolysis. Furthermore, our results are in good agreement with the previously reported capacity of iturin A to form ion-conducting pores in membranes [9] and provide new solid experimental evidence to explain that property of the lipopeptide.

3.3. Apparent size of the iturin-induced membrane lesion

If a colloid-osmotic process is operative, hemolysis should be protected by a solute of the appropriate size added to the outer aqueous solution [20]. The rationale under this consideration is based on the fact that, if the osmotic pressure of intracellular hemoglobin is balanced with another solute added in the outer buffer, which cannot pass through the membrane pore, hemolysis should not occur. In this way, the size of the membrane pore can be estimated by using substances of different sizes. The results obtained for different concentrations of iturin A are shown in Fig. 6. The percentage of hemolysis is plotted against the diameter of the osmotic protectant present in each case. It was found that hemolysis was elicited by compounds having a diameter larger than PEG 4000, independently of the concentration of iturin A. This indicates that the size of the pore is around 32 Å, which is the diameter of PEG 4000. We have no data to give any details about the possible structure of these 'pores'. However, they should be formed by a defined number of iturin A molecules strongly interacting with each other or, given its amphiphilic nature, with neighbouring phospholipid molecules, which is sustained by the great tendency to self-aggregate in the membrane of this lipopeptide [21]. Most probably, this accumulation of molecules of the lipopeptide in a determined region of the membrane would locally enhance membrane permeability to ions (K^+ in this case) thus resulting in a pore behaviour.

3.4. Leakage of liposome contents induced by iturin A

Once it was established that iturin A induced hemolysis of human erythrocytes following a colloid-osmotic mechanism, the question aroused as to whether the lipopeptide was able also to induce contents leakage in liposomes of a define composition. Large unilamellar vesicles (0.1 μm) were prepared with the following compositions: egg PC; POPC; POPC/POPE (1.1); POPC/cholesterol (2:1); some compositions capable of forming membrane lipid rafts [22] like: POPC/POPE/sphingomyelin/cholesterol (2:0.5:2:0.5) and egg PC/sphingomyelin/cholesterol (1:1:1); as well as one resembling the composition of the outer leaflet of erythrocytes membranes, i.e., DOPE/DOPC/sphingomyelin/cholesterol (0.37:0.95:0.16:1.0). In all cases, addition of iturin A at concentrations giving full hemolytic activity resulted in

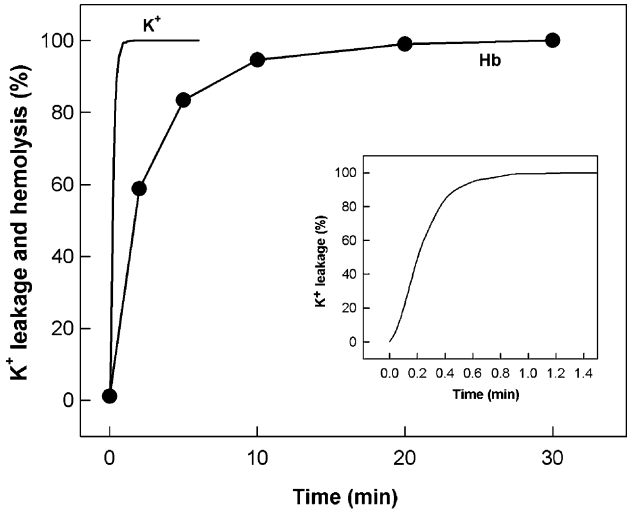

Fig. 5. The relative kinetics of K^+ leakage and hemolysis induced by iturin A. Iturin A was added to a human RBC suspension to a final concentration of 15 μM from a stock solution in DMSO. K^+ leakage was continuously monitored using a K^+-selective electrode. Hemolysis was determined at the indicated times as explained in the text. Temperature was kept at 25 °C using a circulating water bath. The inset shows the first minute of the K^+ efflux curve.

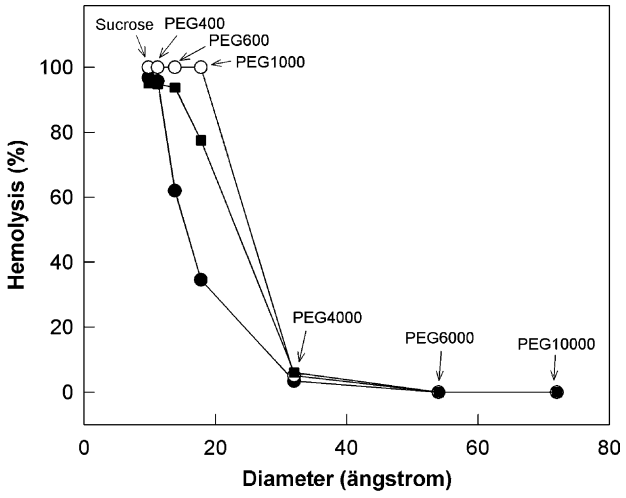

Fig. 6. Iturin A-induced hemolysis in the presence of various colloid-osmotic protectants. Iturin A was added to a human RBC suspension at different final concentrations: 10 (squares), 15 (closed circles) and 20 μM (open circles). Measurements were done at 25 °C. The diameter of the protectants and the details of the hemolysis determination are given in the text.

essentially no effect (results not shown). It is therefore clear that iturin A cannot permeabilize a number of model membranes of quite diverse composition, showing no preference for raft-containing membranes. Furthermore, although it has been reported that iturin A has a strong affinity for artificial membranes containing cholesterol [8], our data indicate that the presence of this compound is not sufficient to allow membrane permeabilization by iturin A. This results could seem apparently in contradiction with the results presented before [9,10] reporting iturin A-induced formation on ion-conducting pores in bimolecular lipid membranes. We think that the discrepancies could be just apparent and due to the very different experimental approaches used, which are not directly comparable since the conditions were completely different. Whereas they measured ion currents (upon voltage application) through planar membranes in 1 M KCl, we are reporting real continuous contents leakage (release of a relatively large molecule like carboxyfluorescein) from large unilamellar phospholipid vesicles, under physiological osmolar conditions (150 mM NaCl).

Then, in order to check whether utilization of intact erythrocytes was a requisite for leakage to be observed, erythrocyte ghosts were prepared containing CF at a self-quenching concentration. Interestingly, addition of iturin A to these erythrocyte ghosts resulted in CF leakage to the external medium (Fig. 7), with a initial rate very close to that observed for iturin A-induced K^+ efflux from intact red cells (Fig. 4, panel A). However, addition of iturin A to CF-containing unilamellar liposomes made with total lipids extracted from erythrocyte ghosts did not induce any leakage (Fig. 7). Our results clearly indicate that whereas iturin A can permeabilize human erythrocytes, releasing potassium and finally resulting in hemolysis, and erythrocyte ghosts, with the concomitant release of trapped CF, it cannot permeabilize unilamellar liposomes of various compositions (including raft-forming compositions), nor liposomes made of erythrocyte membrane lipids, despite the fact that iturin A has a strong tendency to perturb the organization of phospholipid membranes [13]. A possible explanation is that membrane permeabilization induced by iturin A requires the proper accommodation of the lipopeptide in the bilayer to form 'pores', which could rely on the membrane asymmetry, a common characteristic of most biological membranes [23]. Certainly, this is not the only possibility, and the differences between biological and model membranes (once the essential lipid requirements are fulfilled) could be due to a different dependence on the concentration of iturin A. Thus, for model membranes, a higher concentration would be required to achieve the same effect. Unfortunately, the concentration of iturin A cannot go above its CMC since then membrane solubilization starts to occur. Further experimental work, using model membranes, need to be done in order to clarify this issue.

4. Concluding remarks

In this work, we have demonstrated that iturin A causes the hemolysis of human RBC by a colloid-osmotic mechanism. Although the lipopeptide can perturb, the structure of symmetrical phospholipid bilayers, it is not able to permeabilize them. This suggests that the asymmetrical distribution of phospholipids existing in most natural membranes, like the erythrocyte membrane, could be essential for the permeabilizing activity of iturin A, or

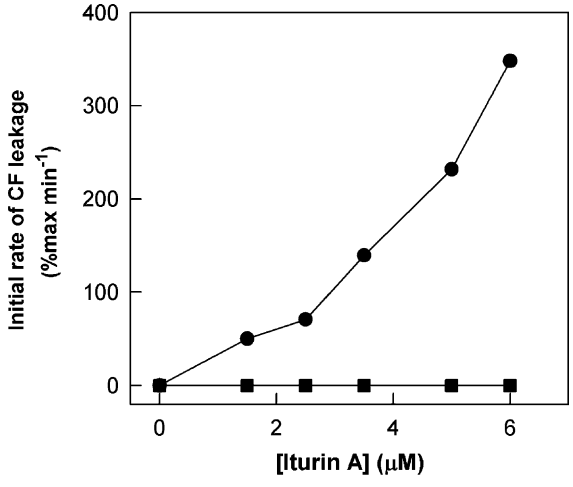

Fig. 7. The effect of iturin A on contents leakage from erythrocyte ghosts and liposomes. Iturin A was added to CF-containing human erythrocyte ghosts (circles) or large unilamellar vesicles made of erythrocyte membrane lipids (squares) and leakage was followed by fluorescence as explained in the text. Phospholipid phosphorus concentration was 25 μM. Temperature was kept to 25 °C.

simply that a higher concentration of iturin A is necessary in model membranes to achieve the same effect as in biological membranes.

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