undergo energetic collisions with argon in Q4, while Q5 was scanned to record the sequential product ion spectrum. Typical reaction energy in Q2 and collision energy in Q4 are 0 eV and 10 eV (under multiple-collision conditions), respectively. All compounds are commercially available and were used without purification.

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Ion-Channel Gating in Transmembrane Receptor Proteins: Functional Activity in Tethered Lipid Membranes**

Thierry Stora, Jeremy H. Lakey, and Horst Vogel*

In memory of Fritz Jähnig

The opening and closing (gating) of ion channels by ligand binding or by changes in the electrical transmembrane potential is the basis of many cellular signal-transduction processes.[1] Furthermore it is of great pharmaceutical importance that the activity of membrane channels can be modulated by the binding of therapeutic agents and thus the understanding of these molecular interactions is a significant aspect in rational drug discovery and design. An increasing number of membrane channels are found to act as targets for medicines, and since combinatorial libraries of potential therapeutic compounds are growing rapidly, fast and highly sensitive methods for functional drug screening are required.[2] Traditional methods such as patch clamp for the investigation of the function of channel proteins are poorly suited to high throughput screening. As an alternative, tethered lipid membranes[3–6] offer attractive possibilities in this context as indicated by the detection and modulation of the channel activity of the small antibiotic peptide gramicidin.[7]

Here we probe the function of a well-characterized pore-forming, transmembrane receptor protein by the measurement in situ of both the binding of ligands by surface plasmon resonance (SPR) and subsequent changes of the channel activity by impedance spectroscopy (IS).[8, 9]

The receptor protein OmpF was reconstituted into a fluid lipid bilayer coupled to a gold surface through the sulfur groups of so-called thiolipids[4] present in the bilayer at a defined molar ratio (Figure 1). OmpF is a member of the porin family from the outer membrane of *Escherichia coli*. It functions as a trimer folded in the form of transmembrane β-barrels,[10] which form distinct ion channels.[11] This membrane protein is also the receptor for the antibacterial toxin colicin N.[12] The colicins constitute a large family, which all exert their toxicity by initially binding to their receptor by a central region termed the receptor binding R domain.[13] It has been shown recently that, whilst colicin N binds to detergent-solubilized OmpF with a dissociation constant K_d of 1 μM, the

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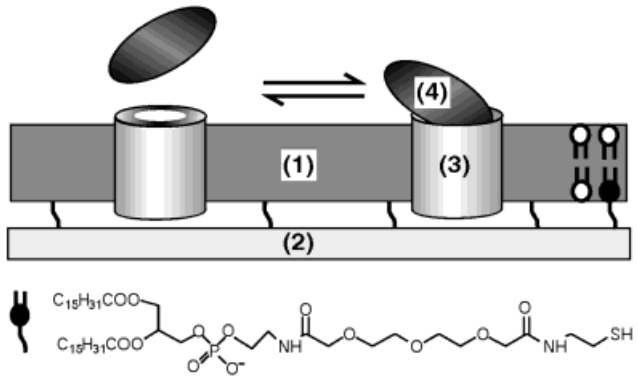

Figure 1. Schematic view of an OmpF-containing lipid bilayer tethered to a gold surface by the synthetic thiolipid *N*-[14′-sulfanyl-1′,11′-dioxo-3′,6′,9′-trioxa-12′-azatetradecyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine shown at the bottom. The thiolipid is designed to form a suitable tethered lipid bilayer for the correct functioning of the reconstituted transmembrane protein:[4] The bilayer can be tuned to an appropriate fluidity and composition, contacts an aqueous environment on both sides of the membrane to accommodate the extramembraneous parts of the protein; a self-assembled monolayer of sulfanylpropionic acid on the gold surface (not indicated) finally prevents all unfavorable interactions between the underlying gold support and the protein. (1) = tethered lipid bilayer, (2) = gold support, (3) = OmpF, (4) = R domain fragment of colicin N.

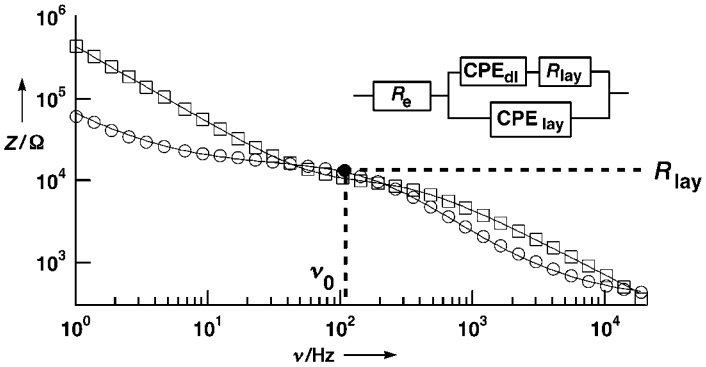

Figure 2. Real (○) and the imaginary (□) part of the electrical impedance spectrum of a 3.14 mm^2 disk gold electrode covered with a supported bilayer: 166 data points equally spaced on the log scale between 1 Hz and 20 kHz were measured, with every fifth point shown. Solid lines represent the fit to the experimental points with the equivalent circuit shown in the top right corner: R_e accounts for the ohmic contributions of the buffer and the counter electrode; the tethered membrane is described by a constant phase element CPE_{lay} (used here as a generalized capacitance) in parallel with a resistance R_{lay} and another generalized capacitance CPE_{dl} in series.[9, 18] The latter two describe the overall contribution of both the membrane-inserted porins and membrane defects. A constant phase element is defined as $1/(j\omega)^n S$, $j^2 = -1$, where ω is the radial frequency, S is the modulus and n the phase; $0 \le n \le 1$, where $n = 0$ corresponds to an ideal resistance, while $n = 1$ corresponds to an ideal capacitance. CPE_{lay} and CPE_{dl} behave approximately like capacitors, as can be seen from the fit values of n_{lay} and n_{dl}. By extraction of the capacitance of the layer from the capacitive, 90° out-of-phase current that arises from CPE_{lay} at the frequency where the phase shift of the whole electrochemical cell is maximum (that is, the current is the most capacitive), we obtained values of 0.9 ± 0.1 μF cm^{-2}. A specific layer resistance of 0.5 – 5 kΩ cm^{-2} was directly deduced (●) without fitting by measurement of the real part of the impedance at the frequency ν_0 where it is the most sensitive to this ohmic contribution (minimum in the phase shift); the relative difference between the value that we got from this procedure and the value obtained from the fit over the whole frequency range was less than 2 %. Typical values for fit parameters are: $R_e = 300 - 500\ \Omega$; $S_{lay} = 1.0 \times 10^{-7}\ \Omega^{-1} s^{n_{lay}}$; $n_{lay} = 0.90 \pm 0.05$; $S_{dl} = 3.0 \times 10^{-7}\ \Omega^{-1} s^{n_{dl}}$; $n_{dl} = 0.95 \pm 0.05$; $R_{lay} = 16 - 160$ kΩ.

isolated R-domain protein fragment exhibited a dissociation constant of greater than 100 μM.[14] This result however did not correlate well with the observed affinity in vivo. In addition, preliminary experiments with black lipid membranes (BLM) revealed that the soluble R-domain fragment could cause closure of the porin channel in lipid bilayers. To obtain physiologically meaningful affinity data we used a tethered bilayer to enable both SPR and IS measurements of the interaction between the R fragment and OmpF.

The tethered lipid bilayers were formed in two steps: Firstly, thiolipids were self-assembled together with sulfanylpropionic acid to create gold surfaces coated with carboxylic acid that were decorated with a certain number of thiolipid anchors. The surface coat of sulfanylpropionic acid prevents the receptor protein from denaturation by direct contact to pure gold. This modified surface was then covered by a bilayer that comprised natural phospholipids and membrane receptors.[15] The whole process was continuously monitored in situ by SPR, which measures the optical layer thickness and thus the amount of surface-immobilized lipid and protein.[16] In addition, the electrical properties of the supported membranes were investigated by IS.[17, 18] The calculated electrical capacitance and resistance of the tethered membranes are in agreement with published data.[4–7] These results confirm that the proteolipid assembly on the gold surface adopts a bilayer membrane structure. Moreover, the conductivity of the membrane $1/R_{lay}$ was measured directly by monitoring the current at one single frequency ν_0 (Figure 2), which is important for the development of the on-line bioassay described below.

OmpF function was probed by independent measurement of its ability to both bind colicin N and form channels that can be modulated by the binding of the ligand. The specific binding of the TR domain of colicin N to the OmpF receptor was measured by SPR (Figure 3).[19] An apparent dissociation constant K_d of 0.6 μM was obtained from the fit to the experimental data. OmpF channel activity was deduced from the membrane conductivity that was monitored by IS. Since ligand-gated ion-channel currents (10^8 ions s^{-1}) provide a considerable intrinsic amplification of the ligand binding,[20] impedance measurements could be carried out with a much lower concentration of receptors in the membranes than for SPR measurements. The data in Figure 3 show that an increase in the R-domain concentration in the bulk solution changes the conductivity of the OmpF channels in the membrane, which reflects functional changes of the receptor upon ligand binding.[21] From these measurements a K_d of 2 μM was evaluated. The relative conductivity changes of 15 % correspond to about 10^6 functional porins in the layer, as deduced from the values reported for the conductivity of a single OmpF porin channel.[11] The data from IS clearly show for the first time that OmpF functions as a ligand-gated ion channel: the binding of the isolated R domain of the toxin on its receptor OmpF closes the ionic channel completely.

This new channel function of the porin, as detected by a novel measurement technique, was corroborated by further use of the well-established BLM technique. OmpF was reconstituted into free-standing bilayers by standard proto-

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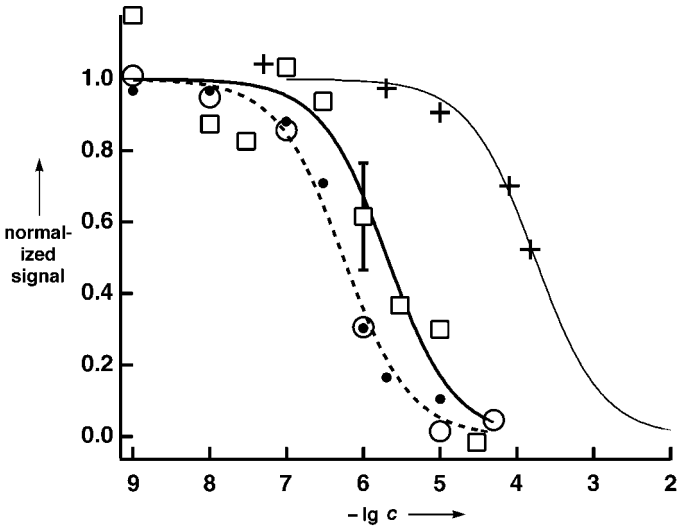

Figure 3. Interaction between colicin N and OmpF. (○): The binding of the colicin TR fragment to its receptor OmpF in a tethered bilayer as measured by SPR. $(\Gamma_{max}-\Gamma)/\Gamma_{max}$ is shown, where Γ and Γ_{max} are the mass of the bound TR fragments at the corresponding and at the highest bulk concentration (that is at saturation), respectively; $\Gamma_{max}=274\ ng\,cm^{-2}$. (□): OmpF channel gating in a tethered bilayer upon R-domain binding measured by IS. Initial value: $1.54\ mS\,cm^{-2}$; final value: $1.35\ mS\,cm^{-2}$. A representative error bar is shown. (●): OmpF channel gating in a free standing bilayer (BLM) upon incubation of the colicin R domain. Initial BLM conductivity: 538 nS; after titration: 0.1 nS. + : Binding of detergent-solubilized OmpF to the surface-immobilized colicin N fragments as monitored by SPR.[9, 18] $(\Gamma_{max}-\Gamma)/\Gamma_{max}$ is shown, where Γ and Γ_{max} are the mass of the bound OmpF at the corresponding concentration and at saturation fitted to the experimental data, respectively. Saturation cannot be obtained experimentally because of the high bulk concentrations of porin required. The value of $808\ ng\,cm^{-2}$ for Γ_{max}, deduced from the fit, corresponds to one OmpF trimer per accessible R domain. Data are fitted by a Langmuir binding isotherm. K_d values of 0.6 and 2 μM are obtained from SPR (○) and IS (□) in the case of tethered bilayers, and 0.5 μM in the case of free-standing bilayers (●), (dashed lines for both SPR and BLM, thick solid line for IS). A K_d value of 170 μM is obtained in the case of OmpF solubilized in detergent. All data sets shown are normalized such that the initial fit values are equal to 1 and the final to 0; the values of the molar concentration c of the ligand in the bulk solution are given as negative decadic logarithms.

cols.[22] The electrical conductivity across the BLM decreased with increasing concentrations of R domain in the surrounding buffer (Figure 3): the OmpF channels were completely blocked at ligand concentrations above 10 μM. From this titration experiment a K_d value of 0.5 μM was obtained. This value confirms the data from IS and SPR of tethered bilayers. Finally, we have determined by SPR that the binding affinity of detergent-solubilized OmpF to surface-immobilized colicin TR domains is about two orders of magnitude lower than that of OmpF reconstituted in lipid bilayers (Figure 3).[23] Again, this is in complete agreement with recently published results[14] and, in the context of our BLM and combined SPR/IS measurements, demonstrates the importance of a lipid bilayer to retain proper native receptor binding activity.

In the context of screening for therapeutic agents, classical binding assays provide only limited information whereas by the combination of SPR and IS we have presented a rapid assay that independently probes both ligand binding and its functional consequences. With the help of nanotechnological fabrication and microfluidic devices,[24] the miniaturization and full automation of this technique opens the door to widespread application for functional screening of ligand–receptor interactions.

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[15] The freshly evaporated gold layer was cleaned by a discharge argon plasma in the vacuum chamber, and immediately incubated for 25 h in a solution of thiolipids and sulfanylpropionic acid (total concentration of $0.5\ mg\,mL^{-1}$ in 48 mM octyl glucoside), at a molar ratio of 1:4 for SPR and 2:1 for IS. The self-assembled monolayer was then washed extensively with a 1% octyl glucoside solution. It was transformed into a bilayer by stepwise dilution of a solution of 48 mM octyl glucoside, $0.5\ mg\,mL^{-1}$ of dioleoyl lecithin, and OmpF ($230\ \mu g\,mL^{-1}$ for SPR and $5\ \mu g\,mL^{-1}$ for IS). Typical thiolipid surface coverage for SPR experiments was 10–25%.
[16] SPR was measured by directing a He-Ne laser light onto a 40 nm thick gold surface through a prism and detecting the dip in the reflected light as a function of the incident angle, as described in the literature.[9] Protein surface coverage Γ is deduced from the SPR angle shift $\Delta\theta$ by using the equation, $\Delta\theta=\alpha\,dn/dc\,\Gamma$ [Eq. (1)], with $\alpha=1.3\times10^{7}\ cm^{-1}$ and a refractive index increment $dn/dc=0.18\ g^{-1}\,cm^{3}$ (*Handbook of Biochemistry*, CRC Press, Boca Raton, **1973**). From structural data of OmpF[10] and Equation (1) we find that a full OmpF monolayer gives surface coverage of $714\ ng\,cm^{-2}$ ($\Delta\theta_{OmpF}=1.7°$). This is significantly higher than for a bilayer composed of pure phospatidylcholines ($\Delta\theta_{lipid}=0.6°$). Therefore, the amount of OmpF incorporated can be deduced directly from the layer angle shift $\Delta\theta_{layer}$ by using $\Gamma_{OmpF}=(\Delta\theta_{layer}-\Delta\theta_{lipid})/(\Delta\theta_{OmpF}-\Delta\theta_{lipid})$; this neglects the minor surface coverage occupied by the thiolipids. Typical values for $\Delta\theta_{layer}$ were 0.9°, which gives an estimated 30% of the layer surface occupied by the incorporated OmpF.

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[17] IS was done with the membrane-covered gold electrode as a working electrode against a counter/reference Ag/AgCl electrode in 0.3 M NaCl/20 mM sodium phosphate (pH 7.4). An alternating current voltage (10 mV root mean square amplitude) was applied and the current was recorded with a phase sensitive Lock-In amplifier to measure the complex impedance Z, as described elsewhere.[9, 18]
[18] T. Stora, R. Hovius, Z. Dienes, M. Pachoud, H. Vogel *Langmuir* **1997**, *13*, 5211 – 5214.
[19] The T domain consists of the natural N terminus of colicin N (residues 1 – 67), and together with the R domain (68 – 190) forms half (= TR) of the entire colicin N molecule (1 – 387). TR served as a natural mass tag to amplify SPR signals, and did not affect the binding affinity of the isolated R domain to the OmpF receptors in the tethered membranes. The quantities of bound TR at saturation were higher than the expected amounts deduced from the estimation of the incorporated OmpF.[11, 18] This could be a consequence of a favorable orientation of the membrane receptors, which arises from their asymmetric structure or to an underestimation of the receptor content in the layer that results from approximate values of the refractive index of the proteins. TR adsorption on a tethered membrane made of pure lipids was very limited, which leads to a mass adsorption of less than 12 ng cm^{-2}.
[20] Since the OmpF single-channel conductivity was reported to be 0.8 nS[11] when 10 mV are applied, a current of 8 pA that passes through a porin corresponds to 10^8 ions s^{-1}.
[21] The calculated capacitance of the layer remained constant within experimental errors. No changes in the conductivity were observed on a pure lipid-tethered membrane upon incubation of the R fragment.
[22] OmpF reconstitution into BLMs were performed as described by R. Benz, *Crit. Rev. Biochem.* **1985**, *19*, 145 – 190. Black lipid membranes were formed by painting a hole of 0.6 mm diameter in a Teflon foil with a solution of 10 mg mL^{-1} soya bean lecithin in decane. OmpF was incorporated afterwards by dilution of 1 μL of OmpF (0.125 mg mL^{-1}) in 1% octyl glucoside in the cell and by application of a constant voltage of 250 mV. The current was recorded at a transbilayer potential of 50 mV direct current. Titration was performed in a solution of 0.3 M NaCl/20 mM sodium phosphate (pH 7.4).
[23] R and TR fragments contained hexahistidine sequences on their N termini which served as tags to immobilize the proteins on nitrilotriacetate-covered gold surfaces by following the procedure described in T. A. Keller, C. Duschl, D. Kröger, A.-F. Sévin-Landais, H. Vogel, S. E. Cervigni, P. Dumy, *Supramol. Sci.* **1995**, *2*, 155 – 160. SPR angle shifts of 0.72° and 1.00° (308 and 427 ng cm^{-2}) were obtained. Only minor binding of the protein was observed at concentrations up to 100 μM upon addition of detergent-solubilized OmpF (as also used elsewhere[14]) to the bulk phase. A further experiment was performed to reduce the packing of R at the interface in which 15% of the protein at the surface was the TR fragment immobilized through the histidine tag that was located at the random coil T domain.[14] As a result, the R segments of the TR fragments protrude above the densley packed layer of T fragments. In this way we could measure an interaction between OmpF and R domain in detergent.
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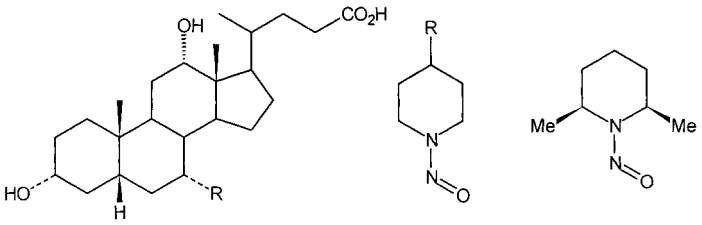
Enantioselective Inclusion Complexation of *N*-Nitrosopiperidines by Steroidal Bile Acids**

Maria Gdaniec,* Maria J. Milewska, and Tadeusz Połoński*

There is currently considerable interest in crystalline inclusion compounds formed by chiral host molecules on account of their application for chiral recognition and optical resolution of racemates.[1] Owing to their ability to accommodate many types of organic guest molecules within their crystal lattices,[2] naturally occuring cholic acid (**CA**) and deoxycholic acid (**DCA**) seem to be very promising hosts for this purpose. However, only very few successful examples of chiral resolution by enclathration with **CA** have been reported,[3] and several attempts to use **DCA** as a resolving agent have failed.[3, 4] On the other hand, we have found recently that the **CA** and **DCA** matrices force conformationally flexible molecules included in the crystals to assume chiral conformations, as indicated by the circular dichroism (CD) spectra.[5]

Here we describe the efficiency of bile acids for chiral recognition of low molecular weight *N*-nitrosamines. The chirality of *N*-nitrosopiperidines **1** – **3** is solely due to hindered rotation of the nitroso group.[6] Because of the partial double bond character of the bond between the two nitrogen atoms in the NNO group, the barrier to rotation about the N–N bond is relatively high (ca. 23 – 25 kcal mol^{-1}).[7] Therefore the enantiomers of **2** and **3** are expected to be stable for a short period of time at ambient temperature even after liberation from the crystal host lattices. Obtaining **1** – **3** in the optically active form



CA, R = OH
DCA, R = H

1, R = H
2, R = Me

3

is challenging from an experimental point of view and would afford very simple models for studying chiroptical spectra of the *N*-nitrosamino chromophore.[8]

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