Biochimica et Biophysica Acta, 367 (1974) 127–133

BBA 76751

TEMPERATURE-DEPENDENT PROPERTIES OF GRAMICIDIN A CHANNELS

E. BAMBERG and P. LÄUGER

Department of Biology, University of Konstanz, 775 Konstanz (G.F.R.)

(Received May 6th, 1974)

SUMMARY

The temperature dependence of the mean life-time and of the conductance Λ of single ion channels induced by gramicidin A in lipid bilayer membranes has been measured. In a second series of experiments, the rate constants of formation (k_R) and dissociation (k_D) of the conducting channel have been determined at different temperatures from electrical relaxation experiments. It has been found that the channel formation proceeds according to a second-order reaction in the whole temperature range (10–40 °C). Furthermore, the mean life-time of the channel approximately agreed with the reciprocal of the dissociation rate constant. Both findings are consistent with the view that the channel consists of a dimer of gramicidin A. From the temperature dependence of Λ, k_R, and k_D the activation energies of ion migration through the channel (E_Λ) as well as the activation energy of formation (E_R) and dissociation (E_D) of the dimer may be calculated. The magnitude of E_D (17 kcal/mole) is consistent with the assumption that dissociation involves the breakage of several hydrogen bonds. The value of E_R (20 kcal/mole) is tentatively explained by the energy required for the structural rearrangement of the lipid matrix in the vicinity of the channel.

Gramicidin A, a linear, hydrophobic pentadecapeptide isolated from *Bacillus brevis*, creates cation-selective channels in lipid bilayer membranes [1–12]. The structure of the channel, as proposed by Urry [13–15], consists of a helix which is formed by head-to-head association of two gramicidin A monomers in the membrane. The $\beta^6{}_{3,3}$-helix has in its center a $4 \cdot 10^{-2}$ μm wide tunnel which is lined with the oxygen atoms of the amide carbonyls. The dimerisation hypothesis is supported by the finding that the covalent dimer which is formed by chemical linkage of two monomers at the formyl ends is even more effective in inducing ion permeability than normal gramicidin A [7, 11]. Further evidence for the dimer mechanism was obtained recently by studying the kinetics of channel formation by electrical relaxation experiments [12]. The results of the relaxation measurements indicate that the channel formation is a second-order reaction; furthermore, they allow a numerical evaluation of the rate constants of formation (k_R) and dissociation (k_D) of the dimer. The aim of the present study was to obtain some insight into the thermodynamics of channel formation. For this purpose relaxation experiments as well as single-channel conductance measurements were performed at different temperatures. From the experimental results

the activation energies for the formation and dissociation of the dimer, the activation energy for the migration of the ion through the channel, and the enthalpy of dimerisation may be obtained.

EXPERIMENTAL

Optical black lipid membranes were formed in the usual way [16] in a thermostated teflon cell filled with 1 M aqueous NaCl solution. The area of the membrane was $8 \cdot 10^{-2}$ or $5 \cdot 10^{-3}$ cm^2 in the case of the relaxation experiments and about $4 \cdot 10^{-4}$ cm^2 in the case of the single-channel conductance measurements. The membrane-forming solution contained 1.0 % (w/v) synthetic, chromatographically pure dioleoyllecithin in n-decane. Two different samples of gramicidin were used, one from the Nutritional Biochemicals Corp. (Cleveland, Ohio), and the other, purified gramicidin A (a mixture of valine and isoleucin analogues), a gift of Dr E. Gross, N.J.H., Bethesda. The commercial product contained in addition to gramicidin A some gramicidin B and C, as well as the analogues of A, B and C in which the N-terminal L-valine is replaced by L-isoleucine. It gave a somewhat broader spectrum of single-channel conductance values than the gramicidin A sample, but otherwise the results were similar. Gramicidin was added from a methanolic stock-solution to the aqueous phase.

The relaxation experiments were carried out as described before [12] by applying a voltage of 135 mV to the membrane through silver–silver chloride electrodes by means of a fast electronic switch. The time course of the membrane current was recorded with a Tectronix 5103 N storage oscilloscope as a voltage drop across an external resistor. The rise-time of the voltage was given by the product of the membrane capacitance and the resistance of the external circuit and was in the range of 1–100 μs.

For the measurement of the current fluctuations arising from the formation and the disappearance of single channels [5, 9] the membrane cell was carefully shielded. The pre-amplifier (Analog Devices Mod. 42 K) was mounted directly above the membrane within the Faraday cage. The signal was further amplified by a Princeton Applied Research Model 113 amplifier and stored on magnetic tape (Precision Instruments, Model PI 6200 recorder) from which it could be transferred on an expanded time-scale to an ordinary strip-chart recorder. In some cases the current fluctuations were directly recorded with a fast ultraviolet recorder (Honeywell 2208 A Visicorder). In order to minimize mechanical vibrations, the membrane chamber was mounted on a large stone-slab which was in turn mounted on an inflated automobile innertube.

RESULTS

An example of the current fluctuations which are observed in the presence of very small amounts of gramicidin A in the aqueous phases is given in Fig. 1. As already described by Hladky and Haydon [9], the amplitudes of the conductance fluctuations are not uniform, but distributed over a certain range. This is shown in Fig. 2 for $T = 10$ °C; the shape of the distribution at $T = 25$ °C and $T = 40$ °C is

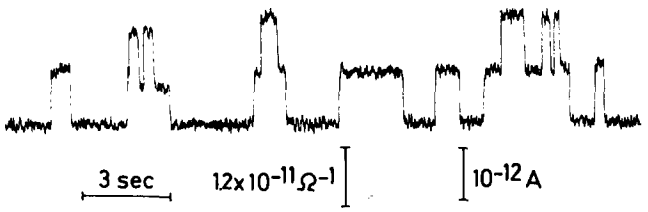

Fig. 1. Discrete fluctuations of the membrane current in the presence of very small amounts of purified gramicidin A. The applied voltage was 90 mV. 1 M NaCl, 25 °C.

similar. The amplitude distribution with the lecithin membranes used in this study is less sharp than the distribution reported by Hladky and Haydon [9] from glyceryl monooleate membranes. It can be seen from Fig. 2 that besides the most probable channel size there is a broad range of channels with smaller conductances. This spread of amplitudes may result from chemical heterogeneity (the sample of gramicidin A used in these experiments contains valine and isoleucin analogues which differ by one methyl group), but we cannot exclude the other possibility that the channel may exist in a number of different conformations.

The single-channel conductance Λ is listed in Table I for three different temperatures. In each case the Λ value is taken at the peak of the amplitude distribution and therefore corresponds to the most frequently occurring channel size. The single-channel conductance at 25 °C is $1.2 \cdot 10^{-11}\ \Omega^{-1}$, whereas Hladky and Haydon [9] observed a value of $2.4 \cdot 10^{-11}\ \Omega^{-1}$ at nearly the same temperature (23 °C) with glyceryl monooleate membranes. The difference in the Λ value may arise from a different geometrical structure of the channel in the two different lipids (dioleoyllecithin and glyceryl monooleate) or from the different surface potential of a dioleoyl-lecithin membrane as compared with a glycerylmonooleate membrane (Anderson, O. S., personal communication).

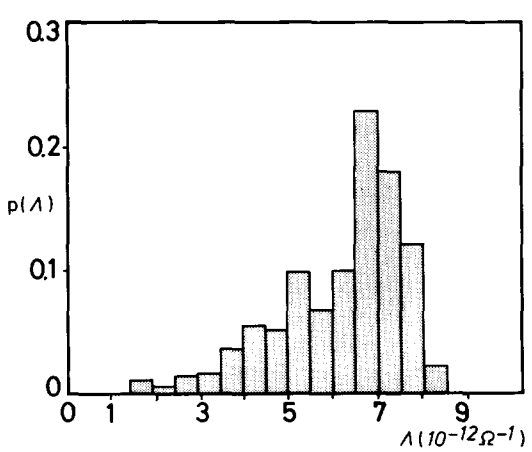

Fig. 2. Probability $p(\Lambda)$ of the occurrence of a conductance fluctuation of magnitude Λ; $p(\Lambda)$ is the number of events falling into an interval of width $\Delta\Lambda = \pm 2 \cdot 10^{-13} \cdot \Omega^{-1}$ centered at Λ, divided by the total number n of events ($n = 685$). Purified gramicidin A, 1 M NaCl, 10 °C.

TABLE I

TEMPERATURE DEPENDENCE OF GRAMICIDIN A CHANNEL PARAMETERS

Dioleoyllecithin in *n*-decane, 1 M NaCl, $V = 135$ mV. T, temperature; k_R and k_D, rate constants of formation and dissociation of the dimer, respectively; K, equilibrium constant of dimerisation; Λ, single-channel conductance; τ_C, mean life-time of a channel, as obtained from the conductance fluctuations. The values of k_R and k_D at 25 °C have been taken from the previous publication [12] and have been corrected for the difference in the applied voltage (135 mV instead of 125 mV in the previous study); in the case of k_D the correction is negligible; k_R has been recalculated using the measured value of Λ at the same temperature (Eqn 3).

T (°C)	k_R ($cm^2 \cdot mole^{-1} \cdot s^{-1}$)	k_D (s^{-1})	$K = k_R/k_D$ ($cm^2 \cdot mole^{-1}$)	Λ (Ω^{-1})	τ_C (s)	$1/k_D$ (s)
10	$2.3 \cdot 10^{13}$	0.25	$8.8 \cdot 10^{13}$	$0.65 \cdot 10^{-11}$	3.8	4.0
25	$20 \cdot 10^{13}$	1.6	$12 \cdot 10^{13}$	$1.2 \cdot 10^{-11}$	1.1	0.63
40	$68 \cdot 10^{13}$	4.5	$15 \cdot 10^{13}$	$2.3 \cdot 10^{-11}$	0.15	0.22

The analysis of the relaxation data has been carried out as described previously [12]. If a voltage is suddenly applied to the membrane, the equilibrium between monomers and dimers

$$A + A \underset{k_D}{\overset{k_R}{\rightleftharpoons}} A_2 \tag{1}$$

is shifted towards a higher dimer concentration. As the current J is proportional to the dimer concentration in the membrane, the approach towards the new equilibrium may be followed by measuring J as a function of time t. By plotting $\ln[(J_\infty - J)/(J_\infty - J_0)]$ against t (J_0 and J_∞ are the initial and the steady-steate current, respectively), it is found that $J(t)$ is governed by a single exponential in the whole temperature range (10–40 °C):

$$J(t) = J_\infty + (J_0 - J_\infty)e^{-t/\tau} \tag{2}$$

The relaxation time τ is related to the rate constants of formation (k_R) and dissociation (k_D) of the dimer in the following way [12]:

$$\frac{1}{\tau} = k_D + 4\sqrt{\frac{k_R k_D \lambda^\infty}{N^0 \Lambda}} \tag{3}$$

where N^0 is Avogadro's number and λ^∞ the steady state conductance which is reached in the limit $t \to \infty$. If $J(t)$ is measured at different gramicidin concentrations and $1/\tau$ plotted as a function of $\sqrt{\lambda^\infty}$, a straight line should result from which the single rate-constants k_R and k_D may be determined. The analysis of the relaxation experiments carried out at 10 °C, 25 °C, and 40 °C gave the expected linear dependence of $1/\tau$ on $\sqrt{\lambda^\infty}$. The values of k_R and k_D obtained in this way are listed in Table I. Also given in Table I are values of the equilibrium constant of dimerisation, $K = k_R/k_D$. It is seen that both k_R and k_D increase markedly with temperature, whereas the temperature dependence of K is rather small.

If the disappearance of a channel corresponds to the dissociation of a dimer, as it is assumed in the model, then the reciprocal of the dissociation rate constant k_D should be equal to the mean life-time τ_C of a channel. Table I shows that the t_C-values which are obtained from the current fluctuations agree with $1/k_D$ within a factor of two, despite a more than twentyfold variation of τ_C and $1/k_D$ in the temperature range 10–40 °C.

DISCUSSION

From the temperature dependence character of the different channel parameters the corresponding activation energies may be calculated. For instance, from $\Lambda(T)$ the activation energy E_Λ of ion migration through the channel is obtained according to

$$E_\Lambda = RT^2 \frac{\mathrm{d} \ln \Lambda}{\mathrm{d}T} \tag{4}$$

(R is the gas constant and T the absolute temperature). The calculated activation energy, $E_\Lambda = 7.3$ kcal/mole (Table II), is somewhat larger than the corresponding

TABLE II

ENERGY PARAMETERS RELATED TO THE GRAMICIDIN A CHANNEL

E_λ, E_Λ, E_R, E_D are the activation energies of the macroscopic membrane conductance λ, the single-channel conductance Λ, the rate constant k_R of formation of the dimer, and the rate constant k_D of dissociation of the dimer, respectively. ΔH, enthalpy of dimerisation (Eqn 5). E_Λ, E_R, E_D refer to 1 M NaCl. All energies are given in kcal/mole.

Lipid	E_λ	E_Λ	E_R	E_D	ΔH
dioleoyllecithin	—	7.3	20	17	3.2
glycerylmonooleate [9]	—	4.5	—	$\lessapprox$ 19	—
soybean lecithin [11]	9.8	—	—	—	—

value of a glyceryl monooleate membrane [9] (4.9 kcal/mole) and about twice as large as the activation energy of diffusion of Na^+ in water (3.6 kcal/mole). The finding that the gramicidin A channel in a dioleoyllecithin membrane has a lower conductance and a higher activation energy as compared with a glyceryl monooleate membrane demonstrates that the properties of the channel may be influenced by the surrounding lipid. Whether the variations of Λ and E_Λ with the lipid reflect a change in the geometrical structure of the channel or merely the difference in the surface potentials [17] is, however, not clear.

The activation energies of k_R and k_D are given in Table II. The activation energy of k_D, $E_D = 17$ kcal/mole, is similar to the activation energy for the termination of the channel in glycerylmonooleate membranes, $E \lessapprox 19$ kcal/mole, as reported by Hladky and Haydon [9]. A value of E_D of the order of 15–20 kcal/mole could account for the energy of several hydrogen bridges and would therefore be consistent with the dimer model of the channel [18]. The activation energy of channel formation, E_R, is

found to be about 20 kcal/mole. A possible explanation of this surprisingly large value may lie in the special geometrical circumstances under which channel formation takes place in the lipid membrane [6, 9]. If the channel consists of a $\beta^6{}_{3,3}$-helix, as proposed by Urry et al. [7], the channel length is 2.5–3.0 nm whereas the hydrophobic thickness of a dioleoyllecithin/n-decane membrane is about 5 nm. The formation of the channel therefore involves a considerable rearrangement of the lipid structure around the dimer, which may require a correspondingly high amount of energy. In view of this interpretation, a measurement of E_R and E_D with lipids of different chain lengths would be interesting. An alternative explanation of the large value of E_R would be that a number of hydrogen bonds between gramicidin and water need to be broken before association can occur.

From the temperature dependence of the equilibrium constant K the enthalpy of channel formation, ΔH, may be calculated according to the thermodynamic relation

$$\Delta H = RT^2 \frac{\mathrm{d}\ln K}{\mathrm{d}T} \tag{5}$$

This yields $\Delta H = 3.2$ kcal/mole. In the light of the tentative interpretation of E_D and E_R given above, the small positive value of ΔH means that the energy liberated by the formation of several hydrogen bonds in the hydrophobic environment is more than compensated by the energy required for the distortion of the lipid matrix.

The activation energy E_λ of the macroscopic membrane conductance λ is related to E_Λ and ΔH in the following way. λ is proportional to the single-channel conductance Λ and to the dimer concentration N_d in the membrane (expressed in moles/cm^2):

$$\lambda = \Lambda N_d N^0 \tag{6}$$

Therefore the relations

$$\frac{\mathrm{d}\ln\lambda}{\mathrm{d}T} = \frac{\mathrm{d}\ln\Lambda}{\mathrm{d}T} + \frac{\mathrm{d}\ln N_d}{\mathrm{d}T} \tag{7}$$

$$E_\lambda = E_\Lambda + RT^2 \frac{\mathrm{d}\ln N_d}{\mathrm{d}T} \tag{8}$$

hold. Introducing the monomer concentration N_m in the membrane:

$$K = \frac{N_d}{N_m^2} \tag{9}$$

and using Eqn 5, one finds:

$$\frac{\Delta H}{RT^2} = \frac{\mathrm{d}\ln N_d}{\mathrm{d}T} - 2\frac{\mathrm{d}\ln N_m}{\mathrm{d}T} \tag{10}$$

In the following, we assume that in measuring $\lambda\,(T)$ the temperature is varied so slowly that a partition equilibrium between the aqueous phases and the membrane always exists. N_m is then related to the aqueous gramicidin concentration c via the partition coefficient β:

$$\beta = \frac{N_m}{c} \tag{11}$$

so that

$$\frac{d\ln\beta}{dT} = \frac{(\Delta H)_p}{RT^2} \approx \frac{d\ln N_m}{dT} \tag{12}$$

where $(\Delta H)_p$ is the enthalpy change associated with the adsorption of one mole gramicidin from the aqueous phase to the membrane. The second part of Eqn 12 implies that the aqueous volume is sufficiently large so that $dc/dT \approx 0$. Eqns 8, 10, and 12 together give

$$E_\lambda = E_\Lambda + \Delta H + 2(\Delta H)_p \tag{13}$$

Thus, if E_λ, E_Λ and ΔH have been determined from the experiments, the enthalpy of adsorption may be calculated. We have not attempted to measure E_λ in the present study, in view of the well-known difficulties to achieve a true partition equilibrium between membrane and water [9]. An experimental value of E_λ, however, has been reported by Goodall [11] (Table II). If it is assumed that a partition equilibrium existed in the experiments of Goodall and if it is further assumed that his lipid (soybean lecithin) is sufficiently similar to dioleoyllecithin, then $(\Delta H)_p$ may be estimated to be of the order of −0.4 kcal/mole. This would mean that the enthalpy of adsorption of gramicidin A is negligibly small.

ACKNOWLEDGMENT

We wish to thank Dr E. Gross for his gift of a sample of gramicidin A. This work has been financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138).

REFERENCES

1 Mueller, P. and Rudin, D. O. (1967) Biochem. Biophys. Res. Commun. 26, 398–404
2 Liberman, E. A. and Topaly, V. P. (1968) Biochim. Biophys. Acta 163, 125–136
3 Tosteson, D. C., Andreoli, T. E., Tiefenberg, M. and Cook, P. (1968) J. Gen. Physiol. 51, 373–384
4 Goodall, M. C. (1970) Biochim. Biophys. Acta 219, 471–478
5 Hladky, S. B. and Haydon, D. A. (1970) Nature 225, 451–453
6 Goodall, M. C. (1971) Arch. Biochem. Biophys. 147, 129–135
7 Urry, D. W., Goodall, M. C., Glickson, J. S., Mayers, D. F. (1971) Proc. Natl. Acad. Sci. U. S. 68, 1907–1911
8 Krasne, S., Eisenman, G. and Szabo, G. (1971) Science 174, 412–415
9 Hladky, S. B. and Haydon, D. A. (1972) Biochim. Biophys. Acta 274, 294–312
10 Myers, V. B. and Haydon, D. A. (1972) Biochim. Biophys. Acta 274, 313–322
11 Goodall, M. C. (1973) Arch. Biochem. Biophys. 157, 514–519
12 Bamberg, E. and Läuger, P. (1973) J. Membrane Biol. 11, 177–194
13 Urry, D. W. (1971) Proc. Natl. Acad. Sci. U. S. 68, 672–676
14 Urry, D. W. (1972) Biochim. Biophys. Acta 265, 115–168
15 Urry, D. W. (1972) Proc. Natl. Acad. Sci. U. S. 69, 1610–1614
16 Läuger, P., Lesslauer, W., Marti, E. and Richter, J. (1967) Biochim. Biophys. Acta 135, 20–32
17 Hladky, S. B. and Haydon, D. A. (1973) Biochim. Biophys. Acta 318, 464–468
18 Haydon, D. A. and Hladky, S. B. (1972) Quart. Rev. Biophys. 2, 187–282