Eur Biophys J (1991) 19:213–216

European Biophysics Journal

Gramicidin in chromatophores of *Rhodobacter sphaeroides*

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Received October 5, 1990/Accepted November 21, 1990

Abstract. Chromatophores of *Rhodobacter sphaeroides* were excited with light flashes to generate a transmembrane electrical potential difference. The electric relaxation was measured by electrochromic absorption changes as a function of added gramicidin. At low gramicidin/bacteriochlorophyll (BChl) molar ratios the decay of the electrochromic absorption changes showed a biphasic behaviour, with a fast phase relaxing at some μs, and a slow phase relaxing at more than 100 ms. This was attributable to a mixture of vesicles containing gramicidin dimers with others containing none. The concentration dependence of this effect was linear. This implied full dimerization of gramicidin. The data were interpreted to yield an average bacteriochlorophyll content per chromatophore of 770(±150) and the conductance of a single gramicidin dimer in the chromatophore membrane of 15(±4) pS (in about 115 mM KCl).

Key words: Photosynthesis – Chromatophore – Gramicidin – Electrochromism – Size analysis

Introduction

The photosynthetic apparatus of certain purple bacteria is organized in invaginations of the plasma membrane, chromatophores, that can be isolated as closed vesicles. In electron micrographs the shape of chromatophores of *Rhodobacter sphaeroides* is approximately spherical with a, somewhat dependent on the preparation, mean vesicle diameter of about 60 nm (Saphon et al. 1975; Packham et al. 1978; Casadio et al. 1988).

Several approaches have been applied to determine the content of BChl of chromatophores: *1*) Centrifugation of a chromatophore suspension of known BChl concentration on to an electron microscope grid and counting vesicles yielded about 5000 BChl/chromatophore (Saphon et al. 1975) and 1000 BChl/chromatophore (Packham et al. 1978) respectively. *2*) Titration with ionophorous antibiotics of the H^+-uptake yielded a BChl/chromatophore ratio of about 400 (Nishimura 1970). Titration with ionophorous antibiotics of the electrochromic carotenoid absorption band shift yielded between 2000 BChl/chromatophore (Nishimura 1970) and 5000 BChl/chromatophore (Saphon et al. 1975), respectively. Valinomycin or nigericin were usually used in these experiments. No published data are available for the acceleration of the decay of the transmembrane voltage in the presence of gramicidin. Instead, it has been claimed that gramicidin "... does not lead to an accelerated carotenoid shift decay" (Saphon et al. 1975). Our experiments showed the contrary. There are several advantages of this channel forming antibiotic over the carrier type ones mentioned above. The much higher conductance of the channel facilitates the kinetic analysis of carotenoid band shifts.

Gramicidin, which is generally accepted to act in biomembranes by head-to-head dimerization of two monomer helices is one of the best-characterized ion pores. Because of its extreme hydrophobicity gramicidin is located practically quantitatively in the membrane (Veatch et al. 1975). A single dimer discharged a chromatophore rapidly in about 10 μs. This allowed a more precise determination of the BChl/chromatophore ratio.

Materials and methods

Chromatophores of *Rhodobacter sphaeroides* (strain ATCC 17023; for growth conditions see Farchaus et al. 1990) were prepared according to Baccarini-Melandri and Melandri (1971) and were stored on ice in the dark for up to 2 days. Chlorophyll determination was carried out in acetone/methanol (7/2, v/v) at 771 nm using a molar extinction coefficient for BChl of ε (771 nm) = $6.85 \cdot 10^{-4}\ M^{-1}\ cm^{-1}$. Flash spectrophotometric exper-

Abbreviations: BChl, Bacteriochlorphyll; tricine, N-Tris[hydroxymethyl]methylglycine

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iments were carried out in a setup as described in Junge (1976). Measurements were performed at room temperature in a cuvette with 2 cm optical path length and 15 ml volume containing 3.5 μM BChl, 94 mM KCl, 7.4 mM $MgCl_2$, 3.1 mM Na-ascorbate, 45 mM tricine/KOH, pH 8.0. After addition of gramicidin the suspension was incubated for 30 min in the dark. The suspension was then excited with saturating flashes of light from a ruby laser ($\lambda = 694$ nm, Q-switch, flash duration about 40 ns, 4 mJ/cm^2) at 5 s intervals. The decay of the electrical potential across the chromatophore membrane was measured via electrochromic absorption changes of intrinsic pigments at 522 nm (Jackson and Crofts 1971; Junge and Jackson 1982). The signal noise ratio was improved by signal averaging. Averaged traces were stored on disk, fitted and plotted on a personal computer. Gramicidin was added to the chromatophore suspension from ethanolic stock solution with vigorous stirring. The ethanol concentration in the cuvette was held below 0.5%. Gramicidin was purchased from Sigma and contained 88% gramicidin A, 7% gramicidin B and 5% gramicidin C, according to the manufacturer.

Results

The spectrophotometric measurements were carried out after 30 min preincubation with gramicidin to reach a homogeneous distribution of the ionophore over the chromatophores. After this preincubation time the signals were stable. Figure 1 shows the time course of the electrochromic absorption change of chromatophores from *Rhodobacter sphaeroides* at various gramicidin concentrations. At lower time resolution (200 μs digitalization time, Fig. 1, top) gramicidin seemingly decreased the extent of the detected signal, without effect on the time course of the remaining transient. At higher time resolution (200 ns digitalization time, Fig. 1, bottom) the apparent loss of the extent at increasing gramicidin concentrations turned out to be a very rapid decay of one portion of the electrochromic absorption change (μs time range). This could be explained in the following way: the chromatophores in the measuring cuvette were divided into two classes: one class carrying at least one gramicidin dimer per chromatophore, and the other class without any gramicidin dimer. The first population, leaky for cations, was responsible for the fast decay in the transients in Fig. 1 (bottom), the second, unaffected, was holding the membrane potential as before. While the initial extent of the carotenoid band shift was constant over the whole concentration range, the amount of the fast phase of the decay increased with the gramicidin concentrations (see Fig. 1, bottom, for 0 nM and 10 nM gramicidin).

Quantitative interpretation of the biphasic decay of the electrochromic absorption changes caused by gramicidin

Averaged transients of electrochromic absorption changes, reflecting the generation and decay of a transmembrane

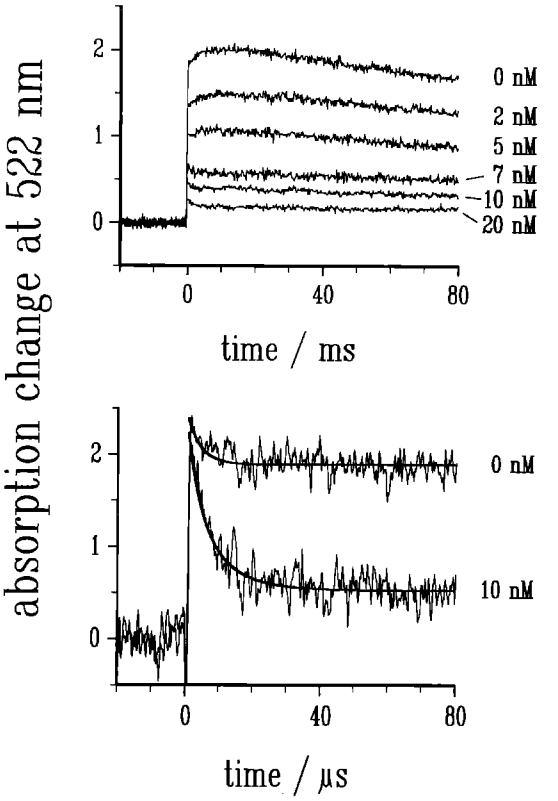

Fig. 1. Electrochromic absorption changes after single flash excitation. Chromatophores were incubated for 30 min in the presence of different gramicidin (monomer) concentrations. The ordinate indicates $-\Delta I/I$ and was scaled up by a factor of 1000. *Top*: 40 transients with 200 μs time resolution were averaged. Gramicidin concentration as indicated. *Bottom*: 120 transients with 200 ns time resolution were averaged. Gramicidin concentration as indicated. The transients were fitted according to Eq. (3) with the following parameters: $n = 0.27$, $k = 0.24\ \mu s^{-1}$ for 0 nM and $n = 1.35$, $k = 0.11\ \mu s^{-1}$ for 10 nM

voltage after a single turnover flash of light, were analyzed with the following assumptions: *1*) The membrane of each chromatophore in the measuring light beam has the same starting voltage after the excitation flash. *2*) The membrane capacitance is discharged only by ion fluxes through gramicidin. *3*) Gramicidin is quantitative dissolved and dimerized in the chromatophore membrane. *4*) Gramicidin dimers behave as ohmic channels. In any given chromatophore the voltage then decays monoexponentially:

$$U(t) = U_0 \cdot \exp(-m \cdot k \cdot t) \quad (1)$$

where k is the decay rate caused by a single gramicidin dimer, m the number of gramicidin dimers in the chromatophore and U_0 the starting voltage. In a concentration range of gramicidin where the number of chromatophores in the cuvette is comparable to the total number of gramicidin dimers, the probability to find m channels per chromatophore, $P(m)$, can be described mathematically by Poisson's distribution:

$$P(m) = (n^m \cdot \exp(-n))/m! \quad (2)$$

where n is the average number of channels per chromatophore. The monitoring light beam in the spectrophoto-

meter probes a large number of chromatophores and the observed decay of the electrochromic absorption changes can be interpreted as the superimposed voltage decay of all chromatophores. As described previously in (Schmid and Junge 1975; Apell and Läuger 1986) combination of (2) with (1) and summation over all m yields:

$$U(t) = U_0 \cdot \exp(-n) \cdot \exp(n \cdot \exp(-k \cdot t)) \quad (3)$$

This equation, which has only two fit parameters, n and k, was used to fit the decay of the observed transients of electrochromic absorption changes that were recorded with higher time resolution (see Fig. 1, bottom). A more rigorous treatment that included the vesicle size distribution has been presented elsewhere (Lill et al. 1987). It did not lead to substantially different results.

The size of chromatophores of Rhodobacter sphaeroides

Figure 2 shows the result of fitting experimental decay curves (as in Fig. 1, bottom) by (3) to extract the parameters n and k. The upper part gives n, the average number of gramicidin dimers per chromatophore as a function of the gramicidin (monomer) concentration. The data points were described by a straight line. Because gramicidin is known to act as a dimer only, this meant that the dimerization of gramicidin was complete (Veatch et al. 1975). The plot in Fig. 2, upper part, also revealed the particular concentration, where each chromatophore contained one conducting gramicidin dimer on the average. At this point the concentration of gramicidin dimers equalled the concentration of chromatophores. So the number of bacteriochlorophylls per chromatophore was simply:

$$[\text{BChl}] \cdot 2/[\text{gramicidin}\,(n=1)] = \text{BChl/chromatophore} \quad (4)$$

On first inspection of Fig. 2, the gramicidin monomer concentration for $n=1$ was 7 nM and therefore, with a BChl concentration of 3.5 μM, the number of BChl per chromatophore about 1000. However, a correction was necessary.

A linear regression of the data points (solid line in Fig. 2, top, valid for gramicidin concentrations from 0 nM to 20 nM) resulted in

$$n = 0.27(\pm 0.05) + 0.11(\pm 0.015) \cdot [\text{gramicidin}]/\text{nM} \quad (5)$$

Without gramicidin the fitted value for the average number of channels per chromatophore was not zero, but appeared to be $n_0 = 0.27$. This was attributable to a rapid decay phase in the absence of gramicidin. It was due to carotenoid triplets also formed by single flash excitation (Wolff and Witt 1969; Kramer and Mathis 1980). The formation of carotenoid triplets enhances the absorption at 522 nm. Their decay occurred in the same time range as the decay of the electrochromic absorption changes when accelerated by gramicidin (see below). While independent of the gramicidin concentration, n_0 could be substracted from the fitted values of n to obtain the true average number of gramicidin dimers per chromatophore. After addition of 9.1 nM gramicidin (monomers) n increased from 0.27 to 1.27. So at 9.1 nM gramicidin (monomers) on the average each chromatophore contained one gramicidin dimer. According to (4), with 4.55 nM gramicidin dimers and 3.5 μM BChl there were 770(±150) BChl/chromatophore.

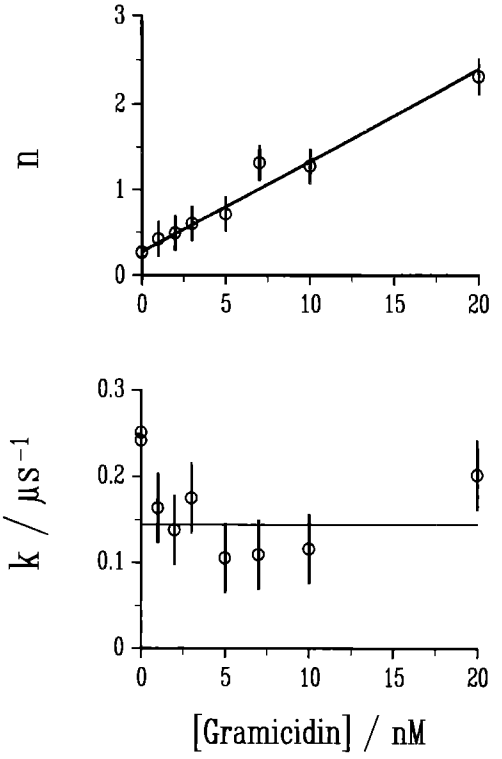

Fig. 2. Results obtained by fitting traces as shown in Fig. 1, lower part. *Top*: Average number of conducting gramicidin dimers per chromatophore, n, as function of gramicidin (monomer) concentration in the chromatophore suspension. Solid line by linear regression of data points (see text). The error bars indicate the standard deviation from the mean of the fitted n values. *Bottom*: Decay rate caused by a single gramicidin dimer, k, as function of the gramicidin (monomer) concentration. The solid line indicates the mean k value of the data points in the range from 1 nM to 20 nM gramicidin. The error bars reflect the standard deviation from the mean of the fitted k values from 1 nM to 20 nM gramicidin

The conductance of a single gramicidin dimer in chromatophores

In Fig. 2, lower part, the decay rate of the electrochromic carotenoid shift as caused by a single gramicidin dimer, k, is plotted against the gramicidin concentration. From 1 nM to 20 nM, k was only little dependent on the gramicidin concentration, as expected. At 0 nM gramicidin the measured decay was not attributable to gramicidin-mediated cation flow but, as mentioned, to the triplet state of carotenoids (see above). The decay rate of this absorption change was 0.25 μs^{-1} which is in good agreement with published data 0.23 μs^{-1} (Wolff and Witt 1969); 0.34 μs^{-1} (Kramer and Mathis 1980). The average value of k (the decay rate caused by a single gramicidin dimer) in the range from 1 nM to 20 nM was 0.14 (±0.04) μs^{-1} (solid line in Fig. 2, bottom). From this value the conductance of a single gramicidin dimer, G,

was calculated according to:

$$k = G/A \cdot C \quad (6)$$

where A is the chromatophore surface area and C the specific capacitance of the chromatophore membrane. Taking 1.1 $\mu F/cm^2$ for C (Packham et al. 1978; Casadio et al. 1988), and $1 \cdot 10^4\ nm^2$ for A (Saphon et al. 1975; Casadio et al. 1988) the conductance of a single gramicidin dimer was 15($\pm$4) pS. With a KCl concentration in our experiments of about 115 mM, this value compares well with the single channel conductance of gramicidin as determined electrophysiologically (14 pS (Hladky and Haydon 1972), 12.3 pS (Neher et al. 1978) in 100 mM KCl.

Discussion

In contrast to earlier studies (Saphon et al. 1975), we found that gramicidin accelerated the decay of the transmembrane electrical potential of the chromatophore membrane. The reported apparent loss of extent of the transients of electrochromic absorption changes at increasing gramicidin concentration turned out to be a rapid decay of one portion of the signal. The "inhibition" by gramicidin of the carotenoid absorbance change reported many years ago (Fleischman and Clayton 1968; Baltscheffsky 1969) was most probably caused by this rapid decay not detected at the then lower time resolution.

Gramicidin forms cation channels as a dimer only (Veatch et al. 1975; Durkin et al. 1990). In the concentration range of our experiments the average number of conducting dimers per chromatophore increased linearly with the gramicidin concentration. Hence, gramicidin was completely dimerized in the chromatophore membrane. In fact, the conductance of a single gramicidin dimer as determined in this work was about the same as the single channel conductance of gramicidin determined electrophysiologically.

The linear dependence between the average number of active ionophores per chromatophore and the gramicidin concentration facilitated a more precise determination of the molar ratio between BChl and gramicidin dimers, and therefore the number of BChl/chromatophore, to 770 ($\pm$150). This value is in agreement with published data from electron microscopy (Packham et al. 1978) and from titration experiments (with ionophores) of the H^+-uptake of chromatophores (Nishimura 1970), but is lower than published values from carotenoid band shift experiments which range up to 5000 BChl/chromatophore (Nishimura 1970; Saphon et al. 1975).

Acknowledgements. We thank Dr. J. Farchaus for providing the bacteria and for the preparation of the chromatophores. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 171/B3).

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