Can a Single Bacteriorhodopsin Molecule Change the Structural State of One Liposome?

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Abstract. Using ultrasonic velocity measurements the interaction of bacteriorhodopsin (BR) with large unilamellar liposomes of dipalmitoylphosphatidylcholine (DPPC) was studied in gel (25 °C) and in liquid crystalline state (50 °C) of lipid bilayer. We could show that with the increasing BR concentration the increment of ultrasonic velocity increases and a saturation occur at a BR/Liposomes of ratio ~ 0.5 mol/mol. BR incorporation into the lipid bilayer in gel state leads to an increase of the increment of the ultrasonic velocity of the lipid to 9.51 ± 1.47 ml/mol. This could be mainly attributed to a decrease in membrane compressibility or an increase in membrane volume or both. No changes of ultrasonic velocity increment were observed with the membrane in liquid crystalline state. In this case, BR probably is not able to change the mechanical properties of a considerably disordered membrane.

Key words: Bacteriorhodopsin — Liposomes — Ultrasonic velocity — Volume compressibility

Investigations of protein-lipid interactions represent an important step towards the understanding of processes of energy transduction by membrane proteins. In particular, it is of importance to know how proteins change the physical properties of the membrane. It is difficult to study the influence of proteins on physical properties of lipid bilayer due to the complex nature of biomembranes. Model systems, liposomes, are useful for this purpose. Liposomes can be modified by proteins and thus their structure becomes similar to that of biomembranes. Of the known energy transducers the integral protein bacteriorhodopsin (BR) is of special interest. BR has a high stability and it can be easily incorporated into liposomes. These properties have made BR useful as a model protein for the study of the interactions of

integral proteins with lipid bilayers. In particular, using fluorescence spectroscopy Rehorek et al. (1985) showed that conformational changes of BR result in an increased ordering of lipid bilayer, and that the transmission of conformational energy covers a distance of more than 4.5 nm. Another parameter, mechanical properties of the membrane, have also been shown to be very sensitive to conformational changes in a lipid bilayer. The influence of BR on the structural state of planar bilayer lipid membranes (BLM) has been demonstrated using a macroscopic method consisting in the measurement of elasticity modulus in direction perpendicular to the membrane plane ($E_\perp$) (Hianik and Vozár 1985). The bilayer area with an altered structure has been shown to exceed 2.8×10^3 nm^2 per one cluster consisting of three BR molecules. Interactions of integral proteins with lipid bilayers can also be investigated by the method of ultrasonic velocity measurements in suspensions of liposomes (Piknová et al. 1991; Tata and Dunn 1992; Hianik et al. 1993). In the present work BR-induced changes of ultrasonic velocity were analyzed in liposome suspensions. The principal experimentally measured value was the concentration increment of ultrasonic velocity, $[u]$, of a liposome suspension, determined by the relation:

$$[u] = (u - u_0)/u_0 c\rho \tag{1}$$

where u is the value of ultrasonic velocity in the liposome suspension; u_0 is the same in pure buffer; c is the molar lipid concentration; ρ is the buffer density. The value of $[u]$ is determined by volume compressibility properties of the liposomes. In diluted solutions, changes of ultrasonic velocity $\delta[u]$ due to some perturbation in the solution (e.g., protein-membrane interaction) are determined by partial molar volume $\delta\overline{V}_0^1$ and partial molar adiabatic compressibility change $\delta\overline{K}_0^1$ (Buckin et al. 1989)

$$\delta[u] = \delta\overline{V}_0^1 - \delta\overline{K}_0^1/2\beta \tag{2}$$

where β is the coefficient of adiabatic compressibility of the solvent.

We studied changes of $[u]$ for liposome suspensions, prepared from dipalmitoylphosphatidylcholine (DPPC), induced by titration with a concentrated solution of purple membrane (PM) fragments containing BR at temperatures below and above the phase transition of DPPC ($T_c \sim 41$ °C).

Experiments were performed on large unilamellar liposomes (approximately 100 nm in diameter), prepared with the method of detergent dialysis as described elsewhere (Piknová et al. 1991). The concentration of liposomes was 2.7 mmol/l. Fragments of PMs containing BR isolated from *Halobacterium halobium* (strain 353P) were added into the liposome suspension. The concentration of BR in the liposome suspension was determined spectrophotometrically using $\varepsilon_{570} = 63000$ mol^{-1}.l.cm^{-1} (Oesterhelt and Hess 1973). As PMs are easily incorporated into liposomes by means of fusion (Vsevolodov 1988) we assumed that the volume concentration of BR was close to that in the liposomes. Due to a very low concentration

of BR we supposed that the incorporation of BR did not substantially change the size of the liposomes. The differential method used allowed us to observe direct effects of BR on the physical properties of the lipid bilayer. PMs were kindly donated by L. N. Chekulayeva. All experiments were done with dark-adapted BR, i.e. the sample was kept in the dark throughout the measurements which started 30 min after the chambers had been filled with the liposome suspension; this is an interval sufficient for BR to become dark-adapted (Stockenius et al. 1979). Ultrasonic velocity measurements were done using the resonator method (Eggers and Funck 1976; Sarvazyan 1982, 1991). The measurements were done at a frequency of approx. 7.2 MHz. The precision of the measurements of relative changes in ultrasonic velocity by the resonator method is of the order of $10^{-4}\%$ (Sarvazyan 1991).

Fig. 1*a* shows the dependence of changes in concentration increment $\delta[u] = [u] - [u]_0$ ($[u]_0$ is the concentration increment in absence of BR; $[u]$ is the same in the presence of BR) on the molar ratio of BR and DPPC liposomes (BR/Liposome) for four independently prepared samples of identical composition, at 25 °C. (The molar concentration of liposomes were calculated from the molar concentration of lipids. The calculations were based on the average diameter of liposomes being 100 nm. Thus, for the average area per one molecule of DPPC of 0.5 nm^2 (Marsh 1990), one liposome contains approximately 1.25×10^5 molecules of lipids). Addition of BR first leads to a growth of parameter $\delta[u]$; from a BR/Liposome ratio of ~ 0.5 mol/mol, the curves reach saturation. Further increasing of BR concentration has no effect on $\delta[u]$. The changes of $\delta[u]$ at the maximum BR/Liposome ratio are 9.51 ± 1.47 ml/mol. No considerable changes in $\delta[u]$ with the increasing BR concentrations were observed at 50 °C (Fig. 1*b*). Let us analyze the changes of ultrasound concentration increment $\delta[u]$ during the incorporation of BR into the liposomes.

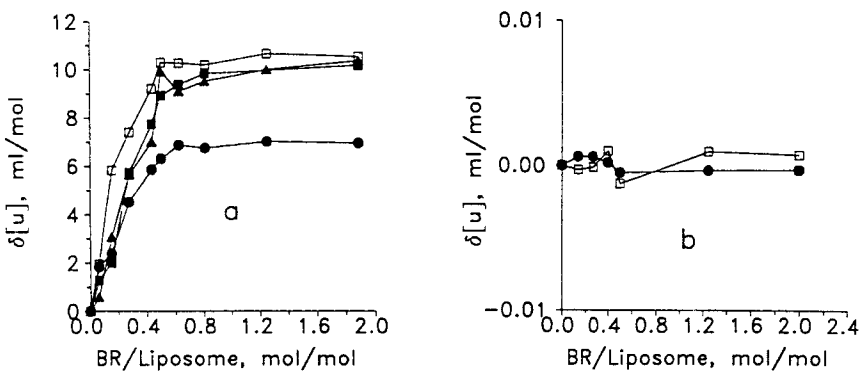

Figure 1. The dependences of concentration increment of ultrasonic velocity changes $\delta[u]$ on molar ratio of bacteriorhodopsin and DPPC liposomes for several independently prepared samples of liposomes of identical composition, at 25 °C (*a*) and 50 °C (*b*).

1. The interaction of BR with liposomes in the lipid bilayer in gel state

a) Stoichiometry of binding

Fig. 1*a* shows that with the lipid bilayer in the gel state, the addition of BR to the liposome suspension raised the ultrasonic velocity increment. This process was completed at the BR/Liposome molecular ratio ~ 0.5 mol/mol. This result can be interpreted in two ways. First, the saturation of the membrane by BR molecules started at a BR/Liposome ratio of ~ 0.5 mol/mol. The increase of $\delta[u]$ and the subsequent saturation of this value can be due to an increase of BR concentration in the membrane and not to long-range interactions of BR with the lipids. Excess BR molecules remain in the solvent. Second, it can be assumed that there is some interaction between BR and the lipid bilayer. At a BR/Liposome ratio of > 0.5 mol/mol, BR is incorporated into the membrane, but this process does not result in changes in volume and in compressibility properties of BR and the membranes. The latter possibility seems to be more appropriate than the former one. Taking into account that 1 PM fragment contains about 10^4 molecules of BR (Vsevolodov 1988), then at a maximal BR/Liposome ratio of 2 mol/mol there actually is but 1 PM fragment per 5000 liposomes.

b) Physical origin of $\delta[u]$ changes

The value of $\delta[u]$ for BR-membrane binding is approx. 10 ml per mole of lipid. This value represents a change in the ultrasonic velocity increment of a liposome + BR system as a result of the incorporation of BR from the solvent (buffer) into the lipid membrane. BR is in the fragments of the purple membranes in the solvent. The fragments are about 5 nm thick and their linear dimensions vary from tenths μm to several μm. The fragments consist of 75% BR and approx. 25% lipids (Vsevolodov 1988). BR in purple membranes are organized in trimmers. The number of lipid molecules per one trimmer of BR in the fragment may vary from 12–14 (Tsyganik and Baldwin 1987) to 32–33 (Blaurock 1975). Due to a relatively low content of lipids in PM we assume that the major influence on the physical properties of liposomes comes from BR. The assumption concerning a negligible contribution of the lipids – surrounded BR to changes in $\delta[u]$ is supported also by our previous results obtained using differential scanning calorimetry (Piknová et al. 1991). In this work we did not observe any considerable shift of the phase transition temperature of the liposome suspension upon increasing the concentration of BR up to a BR/DPPC ratio of ~ 6×10^{-2} mol/mol. The question arises what are the processes that underlie the changes of $\delta[u]$ from of 10 ml/mol. To answer this question the physical origin of the values $\overline{V}_0^1$ and $\overline{K}_0^1$ should be discussed. At a small concentration, the value of the apparent molar volume can be represented by a sum of two members,

$$\overline{V}_0^1 = V_m + \Delta V_h \tag{3}$$

where V_m is the intrinsic molar volume of the solute molecules which is inaccessible to the surrounding water molecules, and ΔV_h is the hydration term determined by the change of density of water in the hydration shell of the solute. The change of V_m resulting from the incorporation of BR from the solution into the liposome membrane could be determined by changes in the intrinsic volume of the membrane $\delta(V_m)_{membr}$, in the hydration of the membrane $\delta(\Delta V_m)_{membr}$, in the intrinsic volume of the BR molecule $\delta(V_m)_{br}$, and in the hydration of the BR molecule $\delta(\Delta V_h)_{br}$

$$\delta\overline{V}_0^1 = \delta(V_m)_{membr} + \delta(\Delta V_h)_{membr} + \delta(V_m)_{br} + \delta(\Delta V_h)_{br} \tag{4}$$

The lipid fraction in PM is so small that its contribution to $\delta\overline{V}_0^1$ can virtually be neglected. An analogous equation holds for partial molar compressibility,

$$\delta\overline{K}_0^1 = \delta(K_m)_{membr} + \delta(\Delta K_h)_{membr} + \delta(K_m)_{br} + \delta(\Delta K_h)_{br} \tag{5}$$

where K_m is the intrinsic compressibility (compressibility of volume V_m) and ΔK_h is the hydration contribution resulting from the compressibility changes of water in the hydration shell as a result of solute-solvent interaction. In general, there could be an additional member on the right side of eq. (5). This is the so-called relaxation contribution (Eggers and Funck 1976; Tata and Dunn 1992) determined by the relaxation processes resulting from changes in pressure and temperature in the acoustic wave of the association constant of BR-membrane binding. However, the physical properties of BR and the membrane make no contribution to this relaxation. Such processes can be expected to be rather long, compared with the frequency of 7 MHz at which the measurements of $[u]$ were done in this work. On the other hand, the relaxation time of conformational changes of BR in the dark is too short (Stockenius et al. 1979). Therefore, in the conditions of the measurements, this process can be expected to "frozen", and the relaxation contribution can be neglected. Let us discuss the four terms in eq. (4–5).

Hydration changes of bacteriorhodopsin molecules

There are no reasons to expect sufficient hydration changes of BR molecules occurring as a result of their incorporation into the lipid bilayer: in the fragments of purple membranes BR is surrounded, as in the liposome membrane, by lipid molecules. There is another argument suggesting that the hydration changes of BR do not represent at least a dominant process. Normally, in association processes hydration decreases. This results in a negative value of $\delta[u]$ (Buckin 1988) due to the release of hydration water which normally is less compressible than bulk water. We obtained a positive value of $\delta[u]$.

The change of internal volume and compressibility of bacteriorhodopsin

The value of $\delta[u]$ of 10 ml/mol DPPC related to 1g of BR gives a value of 0.15 ml/g for the change in the ultrasonic velocity increment of protein. Most globular proteins have a specific partial volume φ_v at 25 °C in the range of 0.69–0.74 ml/g (Zamyatnin 1984). If the entire value of $\delta[u]$ were determined by changes in the protein volume, the volume of BR would increase by 20% as a result of its incorporation into the liposome membrane. This seems to be too much to be realistic.

The coefficient of intrinsic adiabatic compressibility of the protein globule β is about 1.5×10^{-10} Pa^{-1} (Sarvazyan and Kharakoz 1977). This gives a contribution to the value of the ultrasonic velocity increment of BR $\beta \cdot \varphi_v / 2\beta_0 = 0.12$ ml/g. It is less than the measured effect. Thus, even if BR loses all its compressibility as a result of its transport into the membrane, it will not explain the measured value of $\delta[u]$. This means that even if there is a contribution to $\delta[u]$ from changes in internal compressibility and volume of BR, it is not significant.

Change of membrane internal volume and compressibility

The volume occupied by DPPC molecules in a lipid membrane is approx. 700 ml/mol (Marsh 1990). This means that the measured value of $\delta[u]$ corresponds to a BR-binding associated increase in the volume of the lipid membrane by 1.4%. This does not seem unreasonable. For comparison, it should be mentioned that the change in the DPPC membrane volume in the gel-liquid crystalline phase transition is about 5.9% (Marsh 1990). The coefficient of volume compressibility of egg phosphatidylcholine bilayers β is about 6×10^{-10} Pa^{-1} (Buckin et al. 1979). For DPPC membranes, this value is approx. 3.3×10^{-10} Pa^{-1} (Mitaku 1978). The measured $\delta[u]$ recalculated into the change of the coefficient of compressibility gives $\delta\beta = -2\beta_0 \delta[u] / \overline{V}_0^1 = -1.3 \times 10^{-11}$ Pa^{-1}, where $\overline{V}_0^1$ is the molar volume occupied by DPPC in the membrane. This means that a decrease in the membrane compressibility by (2–4)% as a result of BR binding is enough to explain the measured value of $\delta[u]$.

Changes in membrane hydration

As already mentioned, the positive value of $\delta[u]$ corresponds to an increase in hydration. The contribution of hydration to the ultrasonic velocity increment of the components of nucleic acids has been estimated previously (Buckin et al. 1989). These results can be compared with our data. The contribution of hydration to the value $[u]$ for the $-NH_2$ atomic group of adenine is about 2.5 ml/mol. For ribose of nucleosides it is about 20 ml/mol. If the entire value of $\delta[u]$ for BR-membrane binding is a result of a hydration change of the lipid, hydration of every lipid molecule in a liposome would increase by a value corresponding approximately to the hydration of four $-NH_2$ atomic groups or half hydration of ribose. Such high changes in hydration should be accompanied by structural changes in the membrane. On

the other hand, BR has no influence on the thickness of the hydrophobic part of the DPPC bilayer ($\sim$ 3 nm) (Hianik and Vozár 1985, Piknová et al. 1991). It can be expected that even if the molecules of the lipid become hydrated, this is not the dominant process which determines the measured value of $\delta[u]$.

Thus, according to our analysis the main reason for the BR concentration dependent changes in $\delta[u]$ with the membrane in the gel state is the influence of BR on large membrane regions. From the critical BR/Liposome ratio = 0.5 mol/mol it can be assumed that one BR molecule is able to change the physical properties of one liposome. The fact that the BR/Liposome ratio is not an integer quantity may be due to the fact that an average diameter of liposomes of approx. 100 nm was used in the calculation of the molar concentration of liposomes. However, the diameters of liposomes prepared by the detergent dialysis method used in our study show a certain distribution (see Milsmann et al. 1978). Thus we can conclude that the probability of the incorporation of BR into smaller liposomes is higher, and that BR gets first incorporated into liposomes with a smaller diameter.

2. The interaction of bacteriorhodopsin with lipid bilayer in liquid-crystalline state

As already mentioned, at $T = 50$ °C (Fig. 1*b*) there are no changes in the ultrasonic velocity increment of the liposome suspension versus the concentration of BR within the limits of experimental error. This result can be explained as follows: 1. There is no interaction between BR and the liposome membrane; 2. The incorporation of BR from the solution into the membrane does not change the volume and the compressibility properties of the lipid bilayer; 3. There are some changes but, as a result of some compensation effects, they make no contribution to the value of $\delta[u]$. For example, the volume and the compressibility members may give opposite contributions. This would give a zero value of $\delta[u]$ with non-zero $\delta\overline{V}_0^1$ and $\delta\overline{K}_0^1$ values. The first possibility seems unrealistic. A lipid bilayer in the liquid-crystalline state is less ordered than in the gel state (Cevc and Marsh 1987), and as a consequence, it makes the incorporation of BR into the membrane even easier than in the gel state. The second possibility seems more probable. This is also in agreement with the results of Lewis and Engelman (1983) who showed that BR is regularly dispersed in the liquid crystalline state of the membrane and is not able to form any aggregates. Probably, the considerably disordered membrane in this case did not allow BR to make changes in the bilayer structure state.

In conclusion, we could show that: 1. The measurement of the ultrasonic velocity concentration increment $[u]$ is sensitive to the interaction of BR with the lipid bilayer in general, and can be used to study ligand-membrane interaction. 2. The incorporation of BR into the lipid bilayer in the gel state leads to an increase

of $\delta[u] = 10$ ml/mol. This could be attributed mainly to a decrease of membrane compressibility or an increase of membrane volume or both. No changes in $\delta[u]$ were observed in the liquid-crystalline state of the membrane. BR in this case is probably not able to change the structural state of the considerably disordered membrane. 3. To find out more about this result measurements of the volume effect of BR – liposome interaction are required.

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