Electrooptical Measurements on Purple Membrane Containing Bacteriorhodopsin Mutants

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ABSTRACT Electrooptical measurements on purple membrane containing the wild-type and 10 different bacteriorhodopsin mutants have shown that the direction of the permanent electric dipole moment of all these membranes reverses at different pH values in the range 3.2–6.4. The induced dipole moment and the retinal angle exhibit an increased value at these pHs. The results demonstrate that the bacteriorhodopsin protein makes an important contribution to the electrooptical properties of the purple membrane.

INTRODUCTION

Bacteriorhodopsin (bR), the only protein embedded in the purple membrane (pm) from *Halobacterium salinarium* (formerly *halobium*), uses light energy to pump protons (Oesterhelt and Stoeckenius, 1971; Stoeckenius et al., 1979) from the cytoplasmic side to the extracellular side of the cell. In this way, light energy is transformed into electrochemical energy (recent reviews: Henderson et al., 1990; Mathies et al., 1991; Lanyi, 1992; Ebrey, 1993). The charge motion inside the protein causes a displacement current that may be called the protein electric response signal (PERS) (Keszthelyi and Ormos, 1980, 1989; Trissl, 1990).

The current due to the charge motion can be detected only if the pm is oriented, i.e., if one of the sides of the membranes always faces in the same direction. This may happen when the pm is in a suspension or attached to a bilayer. An electric field may orient the membranes through their permanent electric dipole moment and align them via their induced dipole moment in the suspension, or a charged bilayer may attract the membranes.

Electric dichroism permits the observation of the orientation and alignment of membranes in suspension. Measurement of the absorption of light, polarized parallel or perpendicular to the direction of the applied electric fields, as a function of their strength furnishes data on the values of the permanent electric dipole moment (μ) and the polarizability (α) of the pm (Shinar et al., 1977; Keszthelyi, 1980, 1982; Druckmann and Ottolenghi, 1981; Kimura et al., 1981; Tsuji and Neumann, 1981; Todorov et al., 1982; Otomo et al., 1986). The pH dependencies of the permanent dipole moment, the polarizability and the angle of the retinal relative to the membrane normal (θ), which can be determined from these measurements, have already been studied in the case of wild-type bR (Kimura et al., 1981; Barabás et al., 1983). Barabás et al. (1983) recorded the electric signal due to the light-driven proton translocation (PERS) and found that μ reverses its direction in the membrane at around pH 5.0.

The study of charge motion is also very important in the bR mutants (Butt et al., 1989; Otto et al., 1990; Dunach et al., 1990; Gergely and Váró, 1992; Tittor et al., 1994). It seemed worthwhile therefore to attempt to determine the pH dependence of μ, α, and θ also for pm containing bR mutants.

In this study we have determined these parameters of 11 pm-s, each containing different bR mutants (including the wild-type bR), and found for all of them that the sign of μ changes from negative (at lower pH) to positive (at higher pH), but at very different pH values. We define the sign of the permanent dipole moment related to pm as positive if it is directed toward the extracellular side. We also observed that the values of α and θ display resonance-like increases at the pH where μ changes sign.

MATERIALS AND METHODS

The mutants D85N, D85T, D96N, D115N, D212N, D96N/D115N, R82A, R227Q, R82Q/D85N, and T46V, expressed in *Halobacterium salinarium* strain L-33, were supplied by J. Lanyi. We also used the pm from the same wild-type strain. It is assumed that the mutant bRs are in their natural lipid environment in these pm's (Fahmy et al., 1992). After the fragments had been washed three times with tridistilled water to remove sucrose, the optical density of the pm suspended in 1 mM phosphate buffer was adjusted to 1 at 570 nm. The samples were usually gently sonicated for 5 s before the measurements to remove aggregations. Nearly 1 cm^3 suspension was filled in a 1 × 1 cm^2 cuvette. The pH was adjusted with NaOH/HCl and measured with a pH meter (Radelkis OP-208/1, Budapest, Hungary). The conductivities of the samples were set to be the same at all pH values by adding 3 mM NaCl. This was important because the surface pH is greatly affected by the salt concentration (Szundi and Stoeckenius, 1989).

The electric fields (DC and AC) were switched to two platinized platinum electrodes, 1 cm apart, immersed in the suspension, via a homemade programmable switching unit. The DC electric field (0–25 V/cm)

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mainly affected the permanent dipole moment (μ), and the AC electric field (0–300 V/cm at 3.2 kHz) mainly affected the induced dipole moment. These electrodes also served to record PERS.

The light of a 100-W lamp was collimated to pass through a heat filter, a monochromator, and a polarizer. The light intensities (I) through the sample influenced by the electric fields were detected with a photomultiplier tube, amplified with a Keithley-604 differential electrometer amplifier (Keithley Instruments, Cleveland, OH), filtered with a low-pass filter (model 3202; Krohn-Hite Crop, Cambridge, MA) at 10 Hz, and stored in a Thurlby DSA524 digital storage adaptor (Thurlby Electronics, Huntington, England) connected to a computer.

To determine the sign of μ, we measured PERS at all pHs for all of the samples. Laser flashes of the dye rhodamine 6G excited with an excimer laser (Lambda Physik EMG 101 MSC; Lambda Physik, Göttingen, Germany) illuminated the sample in the second half of the DC orientation (the orienting field was ~20 V/cm). The direction of the dipole moment (μ) is related to the first component of PERS (Barabás et al., 1983).

The reduced absorbance change for linearly polarized light (perpendicular or parallel to the direction of the electric field E) is $\Delta A/A = -\frac{1}{A}\log(1 + \Delta I/I)$. Here, ΔI is the change in light intensity due to the field and I is the light intensity without the field. $\Delta A/A$ is generally expressed in terms of an orientation function $\phi(\beta, \gamma)$ (Shah, 1963):

$$\Delta A/A = \frac{1}{2}(3\cos^2\theta - 1)(3\cos^2\sigma - 1)\,\phi(\beta, \gamma), \quad (1)$$

where θ is the angle between the transition dipole moment of the chromophore and the normal of the object (in our case the retinal angle). The term containing σ depends on the direction of the linear polarization of the measuring light relative to the direction of the field. In our work, we used light polarized parallel to the field. The functions β and γ are

$$\beta = \mu E/kT \text{ and } \gamma = \alpha E^2/(2kT)\,, \quad (2)$$

where k is the Boltzmann constant and T is the absolute temperature. The function $\phi(\beta, \gamma)$ is approximated to the following expression:

$$\phi(\beta, \gamma) = \beta^2/15 - 2\gamma/15 - \beta^4/90 - 2\gamma^2/45 + \beta_\gamma^2/30 \cdots; \quad \text{if } \beta, \gamma << 1, \quad (3)$$

We used this equation at very low fields in the range 0.3–1.7 V/cm for the DC field and 5–30 V/cm for the AC field for the calculation of μ and γ from the voltage dependencies of $\Delta A/A$. The retinal angle θ was calculated from the saturation value of $\Delta A/A$ in the case of the AC field when $\phi(\beta, \gamma) = -1/2$.

RESULTS AND DISCUSSION

The pH dependencies of the permanent dipole moment μ, the polarizability α, and the retinal angle θ for the pm containing the wild-type and all of the mutant bRs have been determined in the pH range 3–10. Representative data of the pH dependence for pm containing the wild-type bR, or the mutants D85N, D96N, and R82A, are given in Fig. 1, *a*, *b*, *c*, and *d*, respectively. It may be seen from this figure as well as the results from the other mutants (not shown) that all of the pm's exhibit similar behavior for μ, γ, and θ. The sign of the permanent dipole moment μ changes at a pH (pH_{rev}) different from the value found in wild-type bR (pH_{rev} = 5.2).

The value of pH_{rev} seems to be a characteristic property of the pm because 1) the polarizability α and the retinal angle θ display a broad peak at pH_{rev} at around this pH value; 2) the magnitudes of the parameters μ, α, and θ are different below and above it. Table 1 contains these parameters for the group of pm's studied. It is noteworthy that the highest pH_{rev} was found for the mutant D212N (pH_{rev} = 6.40) and the lowest for D85T (pH_{rev} = 3.20) among the ten mutants investigated.

The asymmetric surface charge of the pm is responsible for the value of μ. The surface of the wild-type bR is more negative on the cytoplasmic side than on the extracellular side above pH_{rev} = 5.2, and less negative below it, as follows from the change of the sign of μ. When the net charge per bR on each side of the pm is calculated as a function of pH from the numerous charged groups of the lipids and the charges on the exposed loops of the wild-type bR, one obtains a reversal of the sign of the difference in the charges between the cytoplasmic side and the extracellular side at about pH 3.5 (Jonas et al., 1990). In this calculation two intramembrane charged residues (D85 and D212), located near the extracellular side, are also taken into account (Table 1 in Jonas et al., 1990). Although the measured pH_{rev} value does not agree with the result of the simple calculation, it is important that the calculation does show that a reversal of the sign of μ (from negative to positive) is expected. The size of the permanent dipole moment, however, is much smaller than what would be expected due to the charged groups. This is probably caused by the cations bound to these charged groups (Jonas et al., 1990).

The observation that pH_{rev} depends on the specific mutation (Table 1) reveals the important role of the bR protein in determining the pH dependence of μ of a pm and consequently its surface charge distribution. The data demonstrate that the charged residues, which are exchanged in mutants, can compete with the other factors that contribute to the value of μ. It is known that the protonation of the aspartic acids depends on pH (Braimann et al., 1988; Gerwert et al., 1989), explaining some of the results, at least qualitatively. It is noteworthy that the pm's containing the mutants D85T and D85N have very different values of pH_{rev}, although a charged residue is exchanged to a neutral one in both cases. Such an anomaly has already been reported for these mutants by Tittor et al. (1994), who noted that the azide anion increased the current pumped by light for the mutant D85N, in contrast with the mutant D85T, which, on the other hand, turned out to be a chloride pump (Sasaki et al., 1995)

The values of μ are smaller below pH_{rev} than above, in accord with the theoretical estimation (Jonas et al., 1990). This information is of practical value: it is more advantageous to orient the pm for measurement of protein electric response signals above pH_{rev}.

The AC field induces a dipole moment in the plane of the pm, just moving the counter-ions along the surface. Thus, the polarizability α depends on the ionic strength and the mobility of the ions at the surface of the pm (Kimura et al., 1981; Papp and Fricsovszky, 1990). Fig. 1 and Table 1 show that the polarizability α exhibits a well-pronounced increase

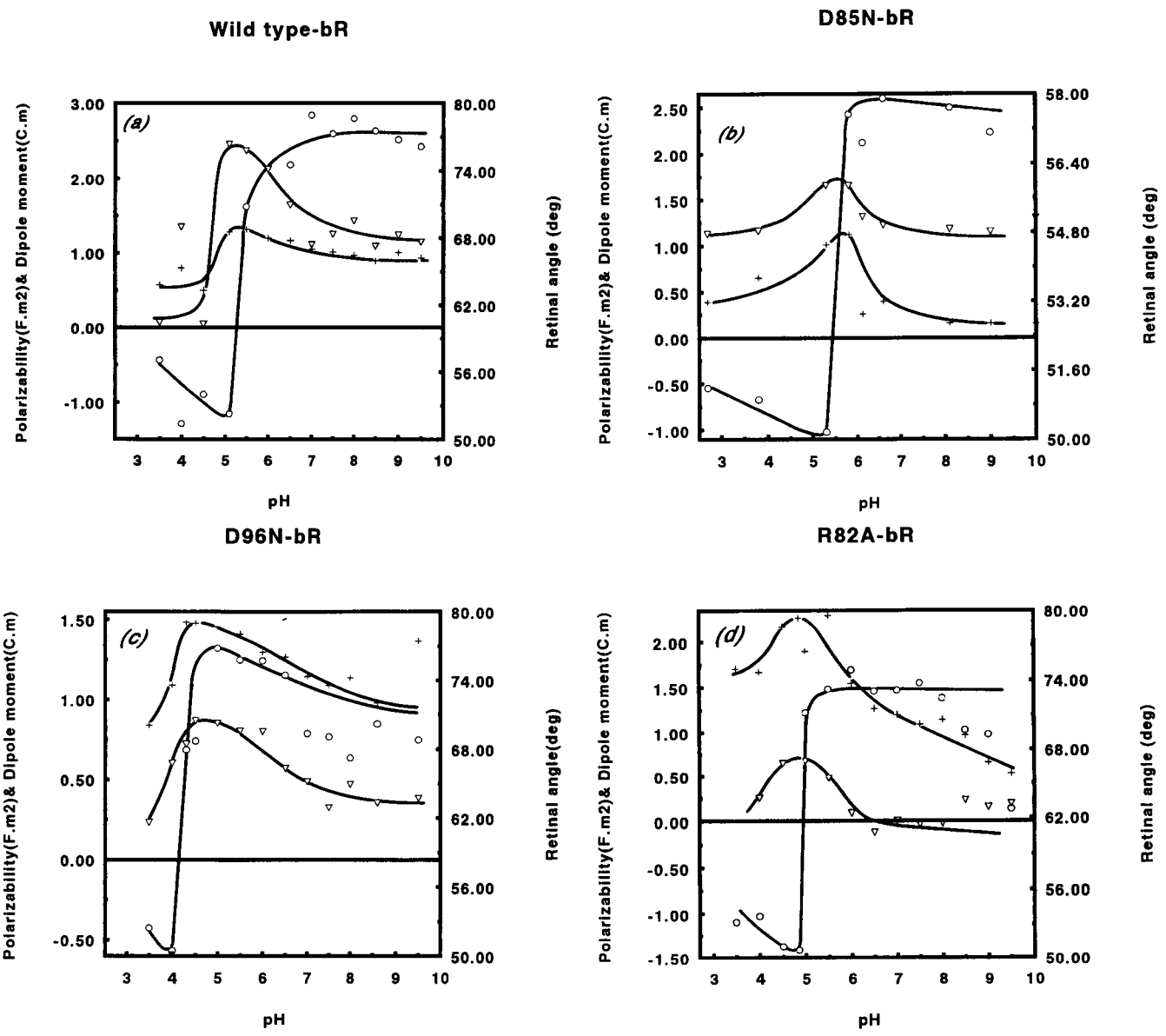

FIGURE 1 pH dependence of dipole moment μ (○, in units of 10^{-23} Cm/membrane), polarizability α (+, in units of 10^{-28} membrane), both on the left axis, and the retinal angle (▽, degrees), on the right axis. (*a*) pm containing wild-type bR; (*b*) pm containing the D85N mutant; (*c*) pm containing the D96N mutant; (*d*) pm containing the R82A mutant.

TABLE 1 The values of pH_{rev}; the permanent dipole moment, in units of 10^{-28} Cm/membrane, above (μ_+) and below (μ_-) pH_{rev}; the polarizability α, in units of 10^{-28} Fm^2/membrane, below (α_-), at (α_n) and above (α_+) pH_{rev}; and the retinal angle θ, in degrees, below (θ_-), at (θ_n) and above (θ_+) pH_{rev}

Sample	pH_{rev}	μ_-	μ_+	α_-	α_o	α_+	θ_-	θ_o	θ_+
D85T	3.20	−1.87	1.10	0.12	0.61	0.22	58.8	60.8	57.8
T46V	3.90	−1.51	1.54	0.82	1.39	0.62	69.0	73.0	65.0
D96N	4.20	−0.43	1.25	0.84	1.50	0.98	61.6	70.5	63.7
R227Q	4.50	−1.36	2.21	0.98	1.92	0.97	60.0	70.9	66.0
D115N	4.60	−0.92	1.70	0.57	1.38	0.55	60.2	70.0	61.0
R82Q/D85N	4.60	−0.32	1.30	0.48	2.06	0.61		69.0	64.0
D96N/D115N	4.65	−1.00	2.60	0.89	1.54	0.64	69.7	72.6	62.0
R82A	4.90	−1.41	1.65	1.66	2.26	1.14	64.0	67.5	61.0
Wild-type	5.20	−1.30	2.60	0.57	1.31	0.93	60.4	76.4	67.6
D85N	5.70	−1.02	2.60	0.40	1.10	0.15	54.76	55.88	54.96
D212N	6.40	−0.50	1.18	1.46	2.02	0.95			64.0

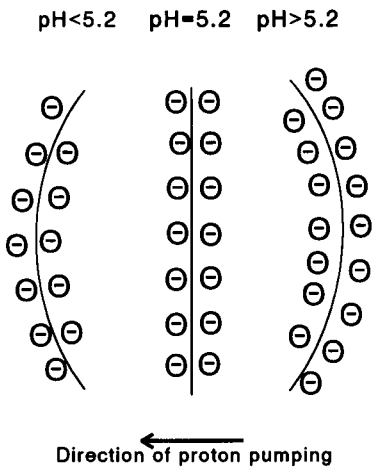

FIGURE 2 Scheme of bending of pm at different pHs.

around pH_{rev} in all cases, as in the case for the wild-type pm (Barabás et al., 1983). The retinal angle θ also displays a maximum around pH_{rev}.

These hitherto undiscussed phenomena seem to be general for all of the pm's studied. It is known that the pm containing the wild-type bR is bent in suspensions (Czégé, 1987a,b; Czégé and Reinisch, 1991). The bending of the pm changes at around pH_{rev} in the case of the pm containing the wild-type bR. Consequently, it should pass through a more or less flat form (Fig. 2). The polarizability α is surely larger for a flat disk than for a bent one because the counter-ions can move a longer distance (Tóth-Boconádi et al., 1994). The average retinal angle θ decreases for both kinds of bent membrane. Our data reveal that this type of bending occurs for pm containing mutant bRs, and it occurs at pH_{rev}.

The magnitudes of μ and α (Table 1) are in the same range as measured earlier for the pm containing the wild-type bR (Kimura et al., 1981; Barabás et al., 1983). However, they do not carry more information, individual for the mutants, because these values depend on the area of the pm and its distribution (Barabás et al., 1983), which were not determined in this study.

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