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Electrooptical analysis of blue and of cation-regenerated bacteriorhodopsin

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Abstract. Blue bacteriorhodopsin was prepared by electrodialysis, cation-exchange chromatography and acidification. The electrooptical properties of these preparations compared to those of the native purple bacteriorhodopsin suggest that the blue bacteriorhodopsin has a smaller induced dipole moment than the native purple bacteriorhodopsin and that bound cations in the native bacteriorhodopsin stabilize the protein conformation in the membrane.

Purple bacteriorhodopsin was regenerated by addition of potassium, magnesium or ferric ions to blue bacteriorhodopsin. Both spectroscopically and electrooptically the potassium- and ferric-regenerated samples are different from the native purple state. Although the magnesium-regenerated sample is spectroscopically similar to the native purple bacteriorhodopsin, the electrooptical properties are rather similar to those of the cation-depleted blue sample, suggesting that it is very difficult to re-stabilize protein structures once cations are depleted.

Key words: Bacteriorhodopsin, blue membrane, purple membrane, electric dichroism, cation binding

Introduction

Upon acidification a reversible colour change of the purple membrane from purple to blue is observed (Oesterhelt and Stoeckenius 1971). The blue membrane is obtained at pH $\approx$ 3, having an absorption maximum at 605 nm. Spectroscopic studies of this acidic blue bacteriorhodopsin suggest a possible relation to one of the intermediates of the photocycle (Moore et al. 1978; Mowery et al. 1979; Tsuji and Rosenheck 1979; Edgerton et al. 1980). Muccio and Cassim (1979) suggested that the blue colour is due to a change in the charge distribution in the local environment of the retinal due to a protonation of an ionizable group.

Recently a blue-coloured membrane was obtained using cation-exchange chromatography by Kimura et al. (1984). Kohl et al. (1984) found that a specific cation binding site in bacteriorhodopsin is important for the native purple colour and the function of the photocycle, by using bacteriorhodopsin containing different cations.

Since the native bacteriorhodopsin undergoes a cyclic reaction by an externally applied electric pulse and since its mechanism is considered to be charge separation of ion pairs – a saturable induced dipole system – in bacteriorhodopsin (Tsuji and Neumann 1981, 1983), electrooptical properties can be expected to be sensitive to the ionic composition of bacteriorhodopsin. In this paper we will describe the purple-blue transition of bacteriorhodopsin by electrodialysis and compare the properties of native bacteriorhodopsin, cation-depleted blue bacteriorhodopsin, as well as the acidic blue bacteriorhodopsin, by means of electric dichroism. In order to know whether this transition is reversible, electrooptical studies of regenerated purple membrane with potassium, magnesium and ferric ions will be reported.

Experimental

Materials

Purple membranes were isolated from *Halobacterium halobium* S9 strain according to Oesterhelt and Stoeckenius (1974). For preparation of the electrically dialyzed blue state of bacteriorhodopsin, 1–2 ml of a purple membrane suspension in water ($OD_{570} \approx 12$) was placed into a dialysis tube. The tube was hung between two platinum electrodes separated by 3–5 cm and the whole system was immersed in quartz-destilled water. A voltage of 50 V was applied

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for 3–5 h, until the blue-purple equilibrium was visibly established. Purple membranes changed colour from purple to blue with a tendency to aggregate. The acidic blue bacteriorhodospin was prepared by adding 0.1 *M* HCl dropwise to a purple membrane suspension of $OD_{570} \approx 0.5$ until the pH of the suspension reached about 3. For the cation-depleted blue bacteriorhodopsin, a purple membrane suspension of $OD_{570} \approx 20$ was passed through a cation exchange column (Bio-Rad, AG-50 W) according to Kimura et al. (1984).

For regeneration of the purple state cation-depleted blue bacteriorhodopsin was used. KCl, $MgCl_2$ and $FeCl_3 \cdot 6H_2O$ of analytical grade were purchased from Merck and used without further purification. The cation-depleted blue bacteriorhodopsin suspension of $\approx 1.5 \times 10^{-5}\,M$ was mixed with the same volume of a $1 \times 10^{-2}\,M$ KCl solution, $1 \times 10^{-3}\,M$ $MgCl_2$ solution or $8 \times 10^{-5}\,M$ $FeCl_3$ solution. Note that the $FeCl_3$ solution was used within 1 minute after dilution from $1 \times 10^{-1}\,M$ $FeCl_3$ solution in order to avoid formation of $Fe(OH)_3$ which does not react with blue bacteriorhodopsin. The mixtures were kept at 4 °C for at least 20 h until a quasi-steady state was established (Zubov et al. 1986). In order to avoid interference with ions all experiments have been carried out in quartz- or polystyrene vessels.

The concentration of purple and blue bacteriorhodopsin was estimated on the basis of $\varepsilon_{565} = 63{,}000$ (Oesterhelt and Hess 1973) and $\varepsilon_{605} = 60{,}000$ (Kimura et al. 1984), respectively. Optical density of the samples for the electrooptical measurements was adjusted to be ≈ 0.4 at the measuring wavelength in order to get sufficiently large signals without serious light scattering contributions.

Absorption measurements

Absorbance spectra were measured with a Cary 219 spectrophotometer (Varian) at 273 K.

Measurements of electric dichroism

The electric dichroism was measured with an electric relaxation spectrometer (Eigen and DeMaeyer 1963; Schallreuter 1982), developed in our laboratory. The time resolution of the detection system is 200 ns. Rectangular electric pulses up to 20 kV/cm and of various duration (10–120 μs) were applied to the samples. The temperature increase due to the Joule heating is negligible (Tsuji and Neumann 1981). For the native and regenerated purple samples the dichroism was measured at 570 nm and for blue samples at 600 nm. All measurements were carried out at 273 K. Since there was a small injection of ions from the measuring cell wall (made of polyvinylchloride), the blue membrane suspensions were replaced after each electric pulse. The colour changed back to purple completely, if the suspension was left in the measuring cell for a few hours.

The change in the absorbancy, ΔA_σ, is calculated from the intensity change of the transmitted light, ΔI_σ, at a given light polarization mode σ,

$$\Delta A_\sigma = -\log(1 + \Delta I_\sigma / I_\sigma), \tag{1}$$

where I_σ is the transmitted light in the absence of the electric field, E. The reduced electric dichroism is defined as

$$\Delta A^{\mathrm{rot}}/A = (A_{\parallel} - A_{\perp})/A, \tag{2}$$

where A is the absorbance at $E = 0$ (Fredericq and Houssier 1973). $\parallel$ and $\perp$ denote that $\sigma = 0$ and $\pi/2$, respectively.

Results

Figure 1 shows the absorption spectra of the native purple membrane suspension in water (pH 6.6), the electrically dialyzed blue membrane suspension in water (pH 5.0), and the acidic blue membrane suspension in water (pH 3.1). When an electric field of 17 V/cm is applied during dialysis, the absorption maximum of the electrically dialyzed membrane shifted (from 570 nm) to 595 nm, indicating that bound cations in bacteriorhodopsin are sensitive to the electric field. The blue state obtained by the cation exchange method (see Fig. 2) has an absorption maximum at 605 nm (Kimura et al. 1984). The purple-blue transition due to electrodialysis apparently was not complete at

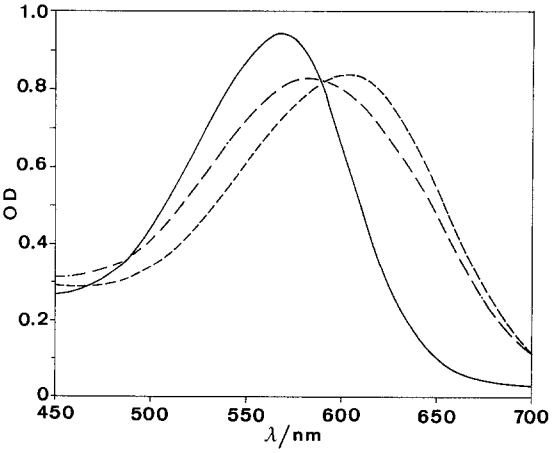

Fig. 1. Absorption spectra of native purple membrane suspension in water at pH 6.6 (——), electrically dialyzed purple membrane suspension in water at pH 5.0 (–·–·–), and acidic purple membrane suspension at pH 3.1 (– – – –)

$E = 17$ V/cm. In the dialysis tube the pH value of the purple membrane suspension changed from 6.1 to 4.5 after a 3 hour-dialysis at 17 V/cm. Upon dilution with water, the pH value increased to pH 5.0, but the blue colour remained.

The colour of the blue membrane returned to purple after addition of cations: the spectra of regenerated purple membrane suspensions in 5×10^{-3} *M* KCl, 5×10^{-4} *M* $MgCl_2$ and 4×10^{-5} *M* $FeCl_3$ are shown in Fig. 2. The corresponding molar ratios of each cation per bacteriorhodopsin were 580 for potassium, 58 for magnesium and 4.7 for ferric ions. The absorption spectrum of the magnesium-regenerated sample is very similar to that of the native sample. On the other hand, the spectra of the potassium- and ferric-regenerated samples are broader than that of the native sample, although the absorption maximum is at 568 nm. This indicates that the native state is not yet reached in these samples. Such a difference in the oscillator strength between the native purple membrane and the regenerated membrane suggests that the blue-purple transition upon addition of cations consists of multiple steps (Kohl et al. 1984; Zubov et al. 1986).

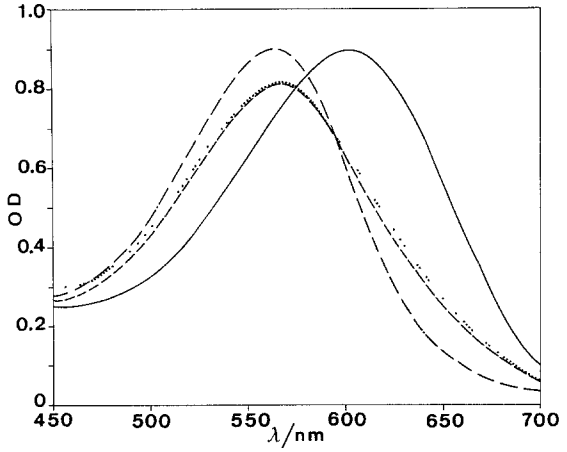

Fig. 2. Absorption spectra of cation-depleted blue membrane suspension (——) and potassium (– – – –), magnesium- (–·–·–) and ferric (····) regenerated samples

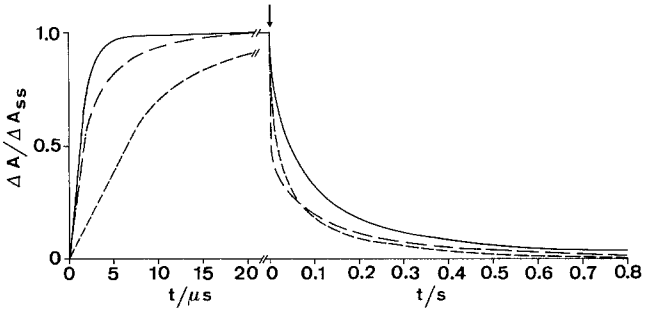

Fig. 3. Time courses of the normalized electric dichroism $\Delta A/\Delta A_{ss}$ for the native purple membrane suspension (——), the electrically dialyzed purple membrane suspension (–·–·–) and the acidic purple membrane suspension (– – – –). ΔA_{ss} is the electric dichroism at the steady state. Arrow shows the time when the electric field was removed; E = 19 kV/cm

Figure 3 shows the response of the electrically dialyzed blue membrane suspension and the acidic blue membrane suspension to a high electric pulse, in terms of the electric dichroism. For comparison the response of the native purple membrane suspension is given. The curves are normalized by the dichroism at the steady state ΔA_{ss}. The average relaxation times obtained by the area method (Yoshioka and Watanabe 1969) are summarized in Table 1. The field-on processes of the electrically dialyzed blue membrane suspension and the acidic blue membrane suspension were 2 and 6 times slower than that of the native purple membrane suspension. The field-off processes of the both blue samples were twice as fast as that of the native sample.

Normalized time courses of the absorption change of the cation-depleted blue sample and the potassium-, magnesium-, and ferric-regenerated samples are shown in Fig. 4. As is seen in Table 1, the relaxation times for both field-on and -off processes of the cation depleted sample are different from the other two blue samples. Among the regenerated samples, the time course of the magnesium sample is the closest to that of the native sample.

The field strength dependence of the electric dichroism for the native purple sample, the electrically dialyzed blue, the acidic blue samples are shown in

Table 1. Mean relaxation time of the field-on and -off processes of the electric dichroism, induced dipole moment ($\Delta\mu$) per reaction unit and electric field strength at $\phi = 1/2$

Sample	Native PM	Blue PM	Acidic PM	Cation-depleted PM	+KCl	+$MgCl_2$	+$FeCl_3$
pH	6.6	5.0	3.1	4.6	4.3	4.2	4.0
$\lambda_{max/nm}$	568	582	602	602	568	565	568
$\tau_{on}/\mu s$	1.5	3.0	9.1	3.9	3.3	2.4	4.6
τ_{off}/ms	130	70	60	100	60	100	50
$\Delta\mu$/Cm	7.0×10^{-26}	3.2×10^{-26}	2.0×10^{-26}	5.1×10^{-26}	2.2×10^{-26}	5.2×10^{-26}	3.7×10^{-26}
/Debye	2.1×10^{4}	9.7×10^{3}	6.0×10^{3}	1.5×10^{4}	6.5×10^{3}	1.5×10^{4}	1.1×10^{4}
$E_{1/2}/Vm^{-1}$	1.6×10^{5}	3.2×10^{5}	5.7×10^{5}	2.1×10^{5}	4.7×10^{5}	1.9×10^{5}	3.0×10^{5}

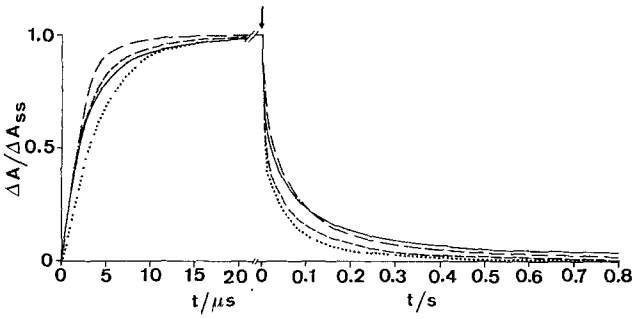

Fig. 4. Time courses of the normalized electric dichroism $\Delta A/\Delta A_{ss}$ for the cation-depleted blue membrane suspension (———) and regenerated purple membrane suspensions with K^+ (– – – –), Mg^{2+} (– · – · –) and Fe^{3+} (· · · · · ·). Arrow shows the time when the electric field was removed; $E = 19$ kV/cm

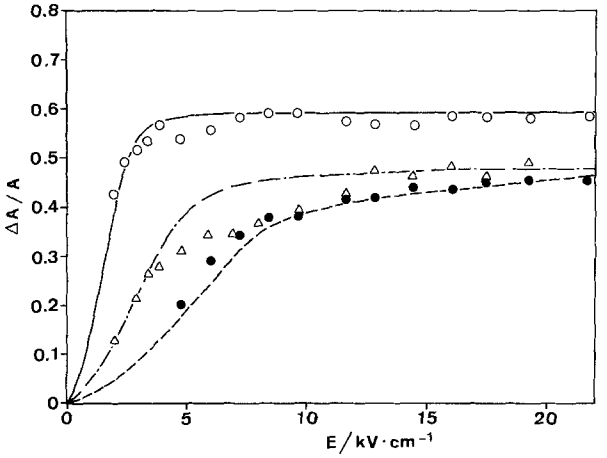

Fig. 5. Electric field strength dependence of the reduced dichroism $\Delta A/A$ for the native purple membrane suspension (○), the electrically dialyzed purple membrane suspension (△) and the acidic purple membrane suspension (●)

Fig. 5: the curve is steeper for the native purple sample, compared to those for the blue samples; at $E = 5$ kV/cm the electric dichroism of the native sample reaches $\approx 100\%$ of the saturation value ΔA_s (the value extrapolated to $E \rightarrow \infty$), while the electrically dialyzed blue sample and the acidic blue sample reaches $\approx 50\%$ and $\approx 30\%$ of their saturation value, respectively. The saturation values for both blue samples are lower than that for the native sample.

Figure 6 illustrates the field strength dependence of the cation-depleted blue membrane suspension and regenerated purple membrane suspensions with potassium ions, magnesium ions and ferric ions. The saturation curve of the cation-depleted blue sample was different from those of the other two blue samples; it was rather similar to that of the native sample, although the initial slope was less steep. The curve for the magnesium regenerated sample was close to that for the native sample, while those for the potassium and ferric regenerated samples were completely different from

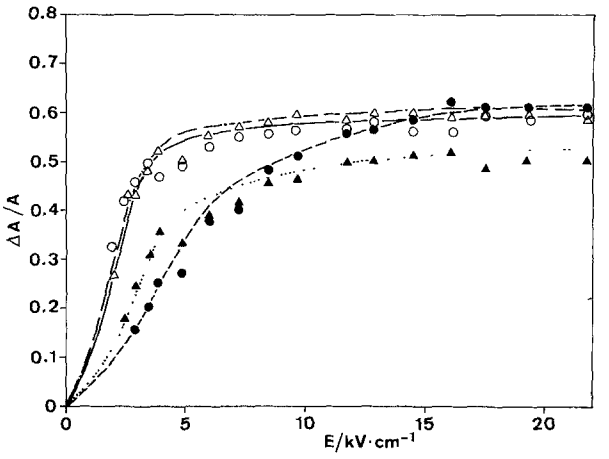

Fig. 6. Electric field strength dependence of the reduced dichroism $\Delta A/A$ for the cation-depleted blue membrane suspension (○) and the regenerated purple membrane suspension with K^+ (●), Mg^{2+} (△) and Fe^{3+} (▲)

that for the native sample – the rise-up is slower and the decay is faster than the native sample.

According to Tsuji and Neumann (1983), the reaction dipole moment ΔM, which causes an equilibrium shift in the presence of the electric field, is roughly estimated from the slope of the field strength dependence of the absorbance change at ϕ $(= \Delta A/\Delta A_s) = 1/2$:

$$\Delta M_{1/2} \approx 4RT\left(\frac{\partial \phi}{\partial E}\right)_{\phi = 1/2, P, T}. \quad (3)$$

The dipole moment difference $\Delta\mu$ per reaction unit involved in the field induced structural transition can be calculated by

$$\Delta\mu = m_1 - m_0 = \Delta M/N_A, \quad (4)$$

where m_0 and m_1 are the dipole moments of the reaction unit in the absence and the presence of the electric field, respectively and N_A is Avogadro's number. The calculated dipole moment difference and the electric field strength $E_{1/2}$ where $\phi = 1/2$ for each samples are listed in Table 1. The rather large value of $\Delta\mu$ (the order of 10^{-26} Cm) suggests that the reaction unit consists of many bacteriorhodopsin molecules. $\Delta\mu$ at $E_{1/2}$ for the blue and acidic blue samples as well as the potassium- and ferric-regenerated samples are smaller than that of the native purple sample, while those of the cation-depleted blue sample and the magnesium regenerated sample are slightly smaller than that of the native sample. Either $\Delta\mu$ per bacteriorhodopsin or the number of the molecules in a reaction unit may be different in each samples.

Discussion

As described above, the electrooptical properties of bacteriorhodopsin depend on its ionic content. Before

we discuss in detail, it should be noted that the fragment orientation is negligible under our experimental condition (Tsuji and Neumann 1981, 1983). In such a case permanent dipoles can not contribute to a total reaction moment, and therefore, $\Delta\mu$ should be an induced-type dipole moment (see Appendix).

The blue state

Among three differently prepared blue samples, the acidic blue sample and the cation-depleted sample are spectroscopically identical (Kimura et al. 1984; Chang et al. 1985), while the electrodialyzed blue sample is an intermediary state of the purple-blue transition (Tsuji and Hess 1986). However, as is shown in Table 1, the electrooptical properties of the acidic blue sample is not identical to those of the cation-depleted blue sample, suggesting that not only an absence of metal ions but also the proton concentration are important factors for dynamic properties of bacteriorhodopsin in the electric field.

Since the electrooptical properties of the native purple membrane are independent of pH in the range of 4.5–7.5 (Tsuji and Neumann 1981), we can consider the difference between the cation-depleted blue sample and the native purple sample as cation effects. The induced dipole moment of this blue state is about 30% smaller than that of the purple state. This means either that the induced dipole moment per bacteriorhodopsin in the blue state is smaller, or that the reaction unit (cooperative unit) is smaller than the native state. The latter case is less realistic, because the two-dimensional crystal lattice is well preserved in the blue state (Kimura et al. 1984). For the former case, there are two possibilities; (*1*) the number of the separable ion pairs is smaller or (*2*) the distance of the separated ion pair is smaller. According to Gerwert et al. (1987) some carboxyl groups of the protein are protonated in the blue state. Since a protonated carboxyl group has less ionic character than a metal-bound carboxyl group, the charge separation, and therefore, the induced dipole moment can be smaller in the blue state.

The field-on process of the conformational change is a complicated function of the induced dipole moment, interacting with the externally applied field, as well as of the fluidity of the local environment where conformational changes are taking place. However, the smaller induced dipole moment for the blue state than for the purple state is qualitatively indicative of the different time courses in the field-on process. On the other hand, since the field-off process reflects the fluidity of the protein in the membrane, it can be concluded that the fluidity of the blue state is higher than that of the purple state. In general, counterions in polyelectrolytes play an important role for stabilizing a molecular conformation. For example, the stacking structure of adenine bases of poly (riboadenylate, K^+) is disturbed by counterion dissociation (Neumann et al. 1983). In this case also, cations weakly bound to the native bacteriorhodopsin may stabilize the conformation of proteins in the membrane.

For the acidic blue bacteriorhodopsin the induced dipole moment is smaller, and the fluidity of the protein is much higher than that of the cation-depleted blue sample, suggesting that excess protons affect the whole membrane system – probably the crystalline lattice is disturbed (Mowery et al. 1979), in contrast to the case with the cation-depleted blue sample.

Behaviour of the electrodialyzed blue sample in the electric field falls somewhat between those of the acidic blue and the cation depleted blue samples. Since not only cations but also anions are removed during electric dialysis, it could be possible that bound anions also contribute to the reaction moment as well as to the stabilization of the protein structure.

Regenerated samples

Potassium- and ferric-regenerated samples show different electooptical properties both from the native sample and the cation-depleted blue samples. Together with spectroscopic measurements and photocycle measurements (Kohl et al. 1984; Chang et al. 1985, 1986), it can be concluded that these samples are far from the native purple state.

Although the purple colour is almost ($> 98\%$) regenerated by magnesium ions (Zubov et al. 1986) and although the photocycle of this magnesium-regenerated sample is similar to that of the native sample (Kohl et al. 1984), the electrooptical properties are rather close to those of the cation-depleted blue bacteriorhodopsin. This suggests either that the remaining colour change of $< 2\%$ is significant for recovering the original induced dipole moment and fluidity, or that the colour transition is not really reversible. It means that it is very difficult or takes a long time to stabilize protein structures, once cations are depleted. It may be due to a rather low pH value ($pH \approx 4.5$) of the magnesium-regenerated sample. However, we have not been able to raise the pH without cation injection.

As a concluding remark we would like to emphasize that electrooptical methods can give information about the reactivity of an ionizable group in the protein in a membrane layer and the effect of the protonation on their structure, which cannot be observed by other methods.

Appendix

Contribution of permanent dipole moments, induced dipole moments and saturated induced dipole moments in a randomly distributed system.

The average contribution of the individual moments $\langle m_j \rangle$ to the total reaction moment is given by

$$\langle m_j \rangle = p_j \langle \cos \theta_j \rangle \tag{A-1}$$

for a permanent dipole system,

$$\langle m_j \rangle = \alpha_j g_j E \langle \cos^2 \theta_j \rangle \tag{A-2}$$

for an induced dipole system and

$$\langle m_j \rangle = (m_s)_j \langle \cos \theta_j \rangle \tag{A-3}$$

for a saturated induced dipole system (Neumann 1986). Here, p_j, α_j and $(m_s)_j$ denote a permanent dipole moment, a polarizability and a saturated induced dipole moment of j-th species, respectively, θ is the angle between the dipole axis and the externally applied electric field and g_j is a conversion factor between the internal field and the externally applied field (Böttcher et al. 1973).

When particles are randomly distributed, the spatial average of cos θ for the permanent dipole system is calculated to be

$$\langle \cos \theta \rangle = \int_0^{\pi} \cos \theta \, 2\pi \sin \theta \, d\theta / \int_0^{\pi} 2\pi \sin \theta \, d\theta = 0. \tag{A-4}$$

This means that randomly distributed permanent dipoles do not contribute to the total reaction moment.

On the other hand, the average for the induced dipole systems is

$$\langle \cos^2 \theta \rangle = 2 \int_0^{\pi/2} \cos^2 \theta \, 2\pi \sin \theta \, d\theta / \int_0^{\pi} 2\pi \sin \theta \, d\theta = \tfrac{1}{3} \tag{A-5}$$

and

$$\langle \cos \theta \rangle = 2 \int_0^{\pi/2} \cos \theta \, 2\pi \sin \theta \, d\theta / \int_0^{\pi} 2\pi \sin \theta \, d\theta = \tfrac{1}{2}, \tag{A-6}$$

indicating that both unsaturated and saturated induced dipoles contribute to the reaction moment in the randomly distributed system.

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