Photochemical Cycle and Light–Dark Adaptation of Monomeric and Aggregated Bacteriorhodopsin in Various Lipid Environments†

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ABSTRACT: Spectral changes of bacteriorhodopsin (BR) reflecting its photochemical cycle and light–dark adaptation were monitored in order to study the effect of protein–protein and protein–lipid interactions on these reactions. For this purpose, the light-driven proton pump BR was reconstituted with various lipids, i.e., dimyristoyl- and dipalmitoylphosphatidylcholine, soybean phospholipids, and diphytanoyllecithin. In these vesicle systems, BR is monomeric above the lipid phase transition and above molar lipid to BR ratios of about 80. Well below the phase transition, BR is aggregated in a hexagonal lattice as in the purple membrane. This allows, on the one hand, comparison of monomeric and aggregated BR in the respective vesicle systems and, on the other hand, comparison of reconstituted BR with BR in the native purple membrane. The photoreaction cycle of *all-trans*-BR accompanying proton translocation proceeds via the same intermediates in the monomeric and aggregated pigment. Furthermore, both the rate and the activation energy for the decay of the cycle intermediate M-410 are independent of the aggregation state. From the results, we conclude that the functional unit responsible for BR's photocycle is the monomer itself. This is in accordance with previous observations that BR monomers are able to translocate protons during illumination [Dencher, N. A., & Heyn, M. P. (1979) *FEBS Lett. 108*, 307–310]. The light–dark adaptation reaction, however, is affected by BR's aggregation state. In the case of the monomer, the extent of light adaptation, i.e., the fraction of BR molecules containing 13-*cis*-retinal as chromophore which is converted by illumination to the respective pigment with the all-trans isomer, is reduced by 50% or more, and the rate of dark adaptation is slowed down about 2.5 times. For these properties too, the monomer is functional, but with a reduced efficiency. This indicates regulatory control by neighboring BR molecules. The rate of the photocycle as well as of dark adaptation is strongly affected by the chemical nature of the lipids used for reconstitution but not by the physical state of the lipid phase.

To understand a membrane system at the molecular level, the structure and function of its building blocks, the proteins and lipids, as well as their mutual interactions have to be known. In the case of the purple membrane (PM)[1] of halobacteria, a membrane system intensively investigated during the last decade (Oesterhelt & Stoeckenius, 1971; Stoeckenius et al., 1979), a study of the protein–protein and protein–lipid interactions seems to be of considerable importance for two reasons. In the first place, the lipids are quite unusual since their hydrocarbon chains are not fatty acids but highly branched saturated 20-carbon isoprenoid alcohols which are ether linked to the glycerol moiety (Kates & Kushwaha, 1976). In the second place, the only membrane protein, bacteriorhodopsin (BR), of the PM is arranged in clusters of three molecules in a two-dimensional hexagonal lattice (Blaurock & Stoeckenius, 1971; Henderson & Unwin, 1975). The light–energy converter BR, a covalently linked retinal–protein complex of molecular weight (M_r) 26 800, is one of the best-characterized membrane proteins. Both the amino acid sequence of the protein moiety (Ovchinnikov et al., 1979; Khorana et al., 1979) and the nucleotide sequence of the respective gene (Dunn et al., 1981) are known. Furthermore, the structure is determined to 3.3-Å resolution (Hayward & Stroud, 1981; Rossmann & Henderson, 1982). Due to the unique arrangement of the BR molecules in the PM, the question of what is the basic functional unit of this light-driven proton pump arose: Is it the lattice, the trimer, or the monomer? The size of the functional unit might even vary for the different reactions catalyzed by BR.

The first approach to answer this question was to disaggregate the PM to the state of BR monomers by means of nonionic detergents and to investigate BR's properties by a variety of biochemical and biophysical methods (Heyn et al., 1975; Reynolds & Stoeckenius, 1977; Dencher & Heyn, 1978; Casadio et al., 1980). The absorption spectrum, secondary structure, and photochemical cycle of BR differed very little in the solubilized and in the native state, indicating that protein–protein interactions affect these properties only in a minor way. Only the extent of light adaptation, i.e., the extent of light-induced conversion of the BR species containing 13-*cis*-retinal as chromophore (13-*cis*-BR) to the respective species with the all-trans isomer (*all-trans*-BR), was found to be altered (Dencher & Heyn, 1978; Casadio et al., 1980). In this experimental approach, the properties of BR monomers in the presence of detergents have to be compared with those of aggregated BR in the PM. Since some of BR's properties are strongly influenced by its environment, this comparison is not unambiguous. Furthermore, it is not possible to investigate the light-driven vectorial proton translocation mediated by BR in these BR–detergent systems.

Reconstituted BR–DMPC vesicles, which allow the reversible disaggregation of BR by merely changing the temperature, are an attractive model system to study protein–protein and protein–lipid interactions (Heyn et al., 1977, 1981a,b;

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[1] Abbreviations: PM, purple membrane; BR, bacteriorhodopsin; BO, bacterioopsin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SBPL, soybean phospholipid(s) (asolectin); DPL, diphytanoyllecithin; CD, circular dichroism; L/BR, molar phospholipid to bacteriorhodopsin ratio; DPH, 1,6-diphenyl-1,3,5-hexatriene; M-410, intermediate with maximal absorbance around 410 nm; Na-DodSO$_4$, sodium dodecyl sulfate.

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Cherry et al., 1978; Casadio & Stoeckenius, 1980). Although it was shown previously that monomeric BR is an effective proton pump (Dencher & Heyn, 1979; Casadio & Dencher, 1981), the effect of BR's state of aggregation on the efficiency of its various functional properties still remained an open question. It was the aim of the present investigation to compare additional features of monomeric and aggregated BR in various reconstituted BR vesicle systems on a quantitative level. The results obtained indicate that the features of the photochemical cycle of *all-trans*-BR accompanying proton translocation are the same or very similar in both aggregation states. No differences were observed in the decay kinetics of the photocycle intermediate M-410 as well as in the respective activation energies between aggregated and monomeric BR. However, both the extent of light adaptation and the rate of dark adaptation depend on the organization of BR. Both quantities are reduced in the case of the BR monomers. Whereas the physical state of the lipids has no influence on the kinetic parameters investigated, the chemical nature of the lipids strongly affects the rates.

Materials and Methods

Chemicals. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and 1,6-diphenyl-1,3,5-hexatriene were obtained from Fluka. DMPC and DPPC showed only a single spot in thin-layer chromatography and were used without further purification. Soybean phospholipids (asolectin) from Sigma were purified as described by Kagawa & Racker (1971). Diphytanoyllecithin was synthesized by K. Janko (Janko & Benz, 1977). Triton X-100 and octyl β-D-glucoside were purchased from Packard and Calbiochem, respectively.

Preparation of Bacteriorhodopsin–Lipid Vesicles. The purified purple membranes (PM) used showed a single band in $NaDodSO_4$–polyacrylamide gel electrophoresis. BR and phospholipid concentrations were determined as previously described (Rehorek & Heyn, 1979). BR was solubilized to the state of monomers in either Triton X-100 or octyl β-D-glucoside (Dencher & Heyn, 1978, 1982). For DMPC and DPPC vesicles, the results were the same with both detergents. BR–SBPL and BR–DPL vesicles with normal spectroscopic properties and with the ability to generate a light-induced proton gradient could only be prepared with octyl glucoside. The monomeric BR was mixed with the respective lipids at the required lipid to BR ratio. In this procedure, the endogenous lipids (about seven per BR) were not removed. During prolonged detergent dialysis, unilamellar vesicles were formed which were purified by sucrose density centrifugation. More details concerning the dialysis procedure and the properties of the reconstituted vesicles can be found elsewhere (Cherry et al., 1978; Heyn et al., 1981a,b; Heyn & Dencher, 1982). The reconstituted vesicles are predominantly unilamellar, with average radii varying between 40 and 500 nm, depending on the vesicle system used, as determined from electron microscopy and quasi-elastic light scattering. All reconstituted vesicle systems used in this investigation were functional and had a net BR orientation. This was inferred from the observation that upon illumination, protons were pumped inward and an alkalization of the medium occurred. Every vesicle preparation was tested in this manner.

Spectroscopy. Absorption spectra and the kinetics of absorbance changes at a fixed wavelength accompanying the so-called light–dark adaptation reaction of BR were recorded in the temperature-controlled scattered transmission accessory of a Cary 118 spectrophotometer. For measurement of the difference spectrum of the photocycle intermediate M-410 which accumulated under continuous illumination, the cuvette with the vesicle suspension was placed in the sample compartment of the photometer and exposed via a flexible light pipe to light from a halogen tungsten lamp (150-W electric power) equipped with a heat filter and a Balzers interference filter (maximal transmission at 543 nm). The light intensity at the surface of the sample was about 2 mW/cm^2, determined with a light detector (Optometer 40X, UDT) connected to a diode (pin 10). The end-on photomultiplier was protected from stray actinic light by means of a Corning CS-7-59 filter. The absorption spectrum of the illuminated vesicle suspension was measured against an identical sample in the dark as reference, preilluminated with the same intensity for several minutes.

The fast transmission changes during the photoreaction cycle of BR were monitored by means of a conventional single-beam flash photometer which had a time resolution of about 10 μs and an amplitude resolution of 10^{-4}, allowing single-shot experiments. The data had a high signal to noise ratio in spite of the fact that single flashes of weak intensity were applied with no signal averaging. The sample cuvette was located very close to the detector to reduce any apparent absorbance changes due to changes in light scattering.

Circular dichroism measurements were performed with a Cary 61 spectropolarimeter modified with a 18-kHz modulator. Fluorescence depolarization of the dye DPH as a function of temperature was recorded by using a Schoeffel RRS 1000 spectrofluorometer with T geometry. The mean vesicle radii of the various reconstituted vesicle preparations were determined by quasi-elastic light scattering (and occassionally by electron microscopy). The latter three techniques were previously described in more detail (Heyn et al., 1981a,b).

Light adaptation was achieved by illuminating the sample for 1 min with light of an intensity of the order of 30 mW/cm^2 and 515 nm $< \lambda <$ 680 nm. The sample temperature was measured in the sample in or slightly above the area of the monitoring beam of the respective photometer by using a very thin copper–constantan thermocouple connected to a digital thermometer (Fluke 2190A). Unless otherwise stated, all experiments were performed in 0.1 M sodium acetate buffer, pH 5.0.

Results

Characterization of Bacteriorhodopsin–Lipid Vesicles. The detergent dialysis method applied in this investigation allows the reconstitution of BR into lipid vesicles without any gross changes in its spectral and functional properties (Cherry et al., 1978; Dencher & Heyn, 1979; Bamberg et al., 1981). As shown in Figures 1 and 2 for BR–DMPC vesicles (L/BR of 94 and 106), the light-adapted pigment has its absorption maximum between 565 and 568 nm at temperatures at which the BR molecules are aggregated (≤10 °C). A 10-nm shift of the absorption maximum to 555–558 nm and a concomitant absorbance decrease of about 13% (corrected for background absorbance) occur upon storage in the dark until spectral equilibration is reached (dark adaptation). Quantitatively the same spectral features are observed for BR in the PM (Oesterhelt et al., 1973; Dencher et al., 1976a). The chemical nature of the lipids does not influence these spectral properties of BR since the same results were obtained also for aggregated BR in a DPPC and SBPL environment.

Although the BR–lipid vesicles prepared by the detergent dialysis method are relatively large, having diameters between 80 and 1000 nm, they are well suited for spectroscopic experiments because of the high transparency of the samples at the vesicle concentrations necessary for the measurements. The

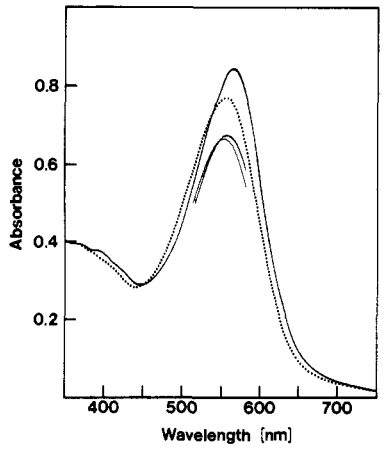

FIGURE 1: Absorption spectra of light-adapted and dark-adapted aggregated and monomeric BR in DMPC vesicles. Upper two spectra: light-adapted (—) and dark-adapted (···) BR–DMPC vesicles at 5.0 °C; BR is hexagonally aggregated. Lower two spectra: the same BR–DMPC vesicles, light adapted (thick curve) and dark adapted (thin curve) at 35.2 °C; BR is monomeric. The spectrum of light-adapted BR at 35.2 °C represents two superimposed traces measured before and after the dark-adapted spectrum was recorded. The vesicles were suspended in 100 mM sodium acetate buffer, pH 5.0. DMPC/BR = 106.

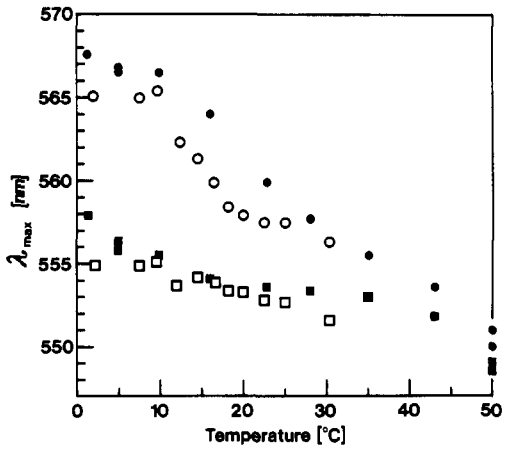

FIGURE 2: Temperature dependence of the absorption maximum of light-adapted (○, ●) and dark-adapted (□, ■) BR–DMPC vesicles. (○, □) DMPC/BR = 94; (●, ■) DMPC/BR = 106.

absorption spectra of BR–DMPC vesicles depicted in Figure 1 were measured by using buffer as reference and are clearly not significantly affected by light-scattering artifacts. Especially in the case of the flash photometric experiments in which the absorbance changes of BR upon excitation by a single flash must be measured without signal averaging, a good signal to noise ratio can only be obtained with clear vesicle suspensions which scatter light only to a minor extent.

Reconstitution of BR into lipid vesicles undergoing a lipid phase transition (e.g., DMPC, DPPC) has the advantage that BR's state of aggregation can be altered reversibly by merely changing the temperature from below the lipid phase transition temperature to above (Cherry et al., 1978; Dencher & Heyn, 1979; Heyn et al., 1981a). The lipid phase transition of pure DMPC vesicles as monitored by the steady-state fluorescence anisotropy of the lipid-soluble fluorescent probe DPH has its midpoint at 23.5 °C (Figure 3B). This value and the sharpness of the transition are an indication that the few detergent molecules which might have remained in the vesicle membrane after dialysis do not severely influence the physical properties of the lipid phase. The same conclusion was reached by using differential scanning calorimetry measurements

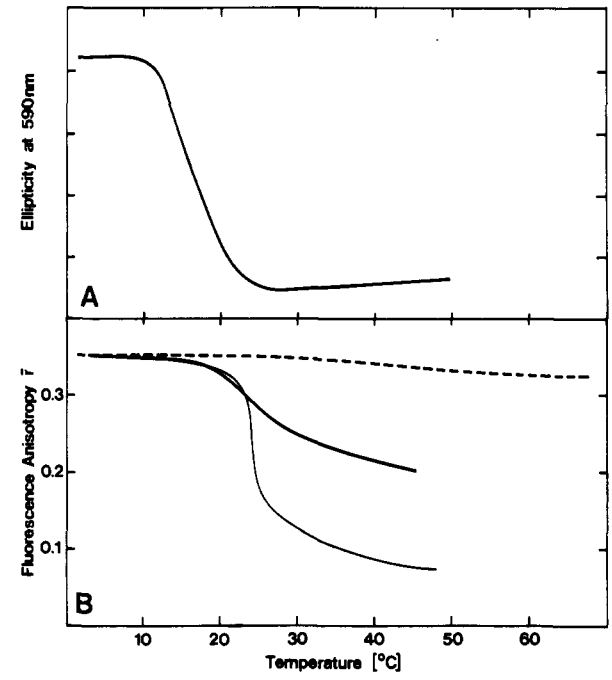

FIGURE 3: Thermotropic behavior of BR–DMPC vesicles, of pure DMPC vesicles, and of purple membranes. (A) Protein aggregation/disaggregation transition in BR–DMPC vesicles as monitored by the temperature dependence of the BR circular dichroism at 590 nm. DMPC/BR = 94. At high temperatures, BR is in the monomeric state; at low temperatures, BR is aggregated. The transition is reversible. (B) Temperature dependence of the steady-state fluorescence anisotropy $\bar{r}$ [$\bar{r} = (F_{\parallel} - F_{\perp})/(F_{\parallel} + 2F_{\perp})$] of DPH embedded in pure DMPC vesicles (—, molar DMPC/DPH ratio of 850), in BR–DMPC vesicles (thick curve, DMPC/BR = 94, DMPC/DPH = 850), and in the PM (---, lipid/DPH = 225).

(Heyn et al., 1981b). Upon incorporation of a large amount of protein into the vesicles (L/BR of 94), the midpoint of the lipid phase transition still remains around 23 °C (Figure 3B). The transition is broadened, however, and the anisotropy of the DPH fluorescence is increased in the liquid-crystalline phase (Figure 3B) (Heyn, 1979; Heyn et al., 1981a,b). In Figure 3B, the temperature dependence of the fluorescence anisotropy of DPH embedded in native PM is included for comparison.

A necessary prerequisite for the experimental approach followed in this report is to have a method available allowing the determination of BR's aggregation state in the vesicles. In previous work, it was shown that BR monomers and aggregates may be distinguished on the basis of their different characteristic CD spectra (Heyn et al., 1975; Bauer et al., 1976; Becher & Ebrey, 1976). For BR–DMPC vesicles far below the lipid phase transition temperature, an exciton CD spectrum was observed which is very similar to that of BR in the PM, indicating that the BR molecules are aggregated in the same spatial arrangement in both systems (Cherry et al., 1978; Heyn et al., 1981a). X-ray diffraction measurements on BR–DMPC vesicles showed that the aggregated BR molecules are crystallized in the same hexagonal lattice as in the PM (Cherry et al., 1978). At temperatures above the lipid phase transition temperature, however, the CD spectrum changes to a broad positive band centered at the absorption maximum. This is typical for monomeric BR since such spectral features are observed in solubilized BR monomers (Heyn et al., 1975; Reynolds & Stoeckenius, 1977; Dencher & Heyn, 1978). For determination of the temperature range in which BR in a DMPC bilayer is aggregated and monomeric, respectively, the temperature dependence of the ellipticity at

590 nm, the wavelength of maximal difference between the CD spectra of both aggregation states, was measured. This CD transition curve (Figure 3A), which monitors the protein crystallization, is characterized by a midpoint at about 16 °C and plateau values at lower and higher temperatures. From these data, it can be concluded that all BR molecules are aggregated below about 10 °C and are monomeric above 23 °C. At around 16 °C, i.e., well below the phase transition temperature of the lipid, the vesicles are composed of a 1 to 1 mixture of aggregated and monomeric BR. In BR–DPPC vesicles, the protein crystallization is also shifted toward lower temperatures as compared to the lipid phase transition (midpoint at 34.5 °C as compared to 41 °C for the pure lipid; data not shown). The conclusions, drawn from the CD measurements are in quantitative agreement with data of the temperature dependence of the rotational diffusion of BR in DMPC vesicles as determined by flash-induced transient linear dichroism (Heyn et al., 1981a). SBPL and DPL are in the liquid-crystalline state at temperatures above 0 °C. In accordance with this, BR was found to be monomeric in vesicles reconstituted with one of these lipids as long as the molar lipid to BR ratio was larger than about 80.

Extent of Light Adaptation. As described in the previous section, aggregated BR in DMPC vesicles undergoes the same spectral changes during light–dark adaptation as BR in the PM. In the PM, these spectral changes reflect changes in the isomeric composition of the chromophore; i.e., in the light-adapted BR (moderate light intensities; Sperling et al., 1979), the chromophore retinal is exclusively in the all-trans configuration whereas the dark-adapted BR is composed of a 1 to 1 mixture of the all-trans and 13-cis isomer (Oesterhelt et al., 1973; Dencher et al., 1976a,b; Ohno et al., 1977; Sperling et al., 1977; Pettei et al., 1977). It was therefore expected that the same correlation between spectrum and isomer composition holds for aggregated BR in the DMPC vesicles. This assumption was verified by measuring the isomer composition by means of a nondestructive flash spectroscopic technique based on the known differences in the photoreaction cycle of 13-*cis*- and *all-trans*-BR (Dencher et al., 1976a; Sperling et al., 1977, 1979). It was found that the chromophore of light-adapted BR in DMPC vesicles at 5 °C, being in the aggregated state according to the CD results of Figure 3A, is solely *all-trans*-retinal. After dark adaptation, however, it has changed to a 1:1 mixture of both isomers (data not shown).

Increasing the temperature to 35 °C does not only result in a pronounced decrease of BR's extinction coefficient but also reduces the spectral differences between the light- and dark-adapted photopigment (Figure 1). At this temperature, BR is monomeric (Figure 3A). As demonstrated by the absorption spectra (Figure 1) and the plot of the temperature dependence of the wavelength of the absorption maxima (Figure 2), the absorption maximum of the light-adapted BR shifts by 11 nm to shorter wavelengths during the temperature increase from 5 to 35 °C as compared to only 3 nm for the dark-adapted pigment. Therefore, the wavelength difference of the absorption maxima of light- and dark-adapted BR is only 2.5 nm at 35 °C compared to 10.5 nm at 5.0 °C. The increase in the extinction coefficient upon illumination of dark adapted BR of about 13% at 5 °C is reduced to 2% at 35 °C (Figure 1). At 23 °C, where the BR molecules are all in the monomeric state (Figure 3A), the corresponding values are 5 nm for the λ_{max} difference (Figure 2) and 5% for the absorbance difference. These alterations are not due to destruction of the chromophore during illumination at elevated temperatures since the spectral changes are fully reversible. The spectrum of light-adapted BR at 35 °C shown in Figure 1 is actually composed of two superimposed traces measured before and after the dark-adapted spectrum was recorded. No differences are visible. The plot of the temperature dependence of the wavelength of the absorption maximum (Figure 2) shows that the maximum of dark-adapted BR in DMPC vesicles shifts only slightly in a monotonous way from about 556 nm at 5.0 °C to 553 nm at 35 °C. The results suggest that the isomeric composition of the chromophore in dark-adapted BR does not alter drastically with increasing temperature and during disaggregation of the protein lattice. Determination of the isomer composition by flash spectroscopy confirmed this hypothesis (data not shown).

In the case of the light-adapted BR, however, a pronounced temperature dependence of λ_{max} was observed. In the temperature range from 1.3 to 10.0 °C, in which all BR molecules are aggregated, λ_{max} is nearly temperature independent but shifts drastically by 8.5 nm from 566 to 557.5 nm between 10 and 20 °C. Further increase in temperature leads to a continuous but less drastic decrease in λ_{max} (Figure 2). Due to the broad absorption band and to the relatively fast rate of dark adaptation at higher temperatures (see below), the exact λ_{max} is difficult to determine. This might explain the slight scattering in the λ_{max} values obtained from two different vesicle preparations with very similar lipid to protein ratios (Figure 2, open and closed circles). In order to circumvent these problems, we calculated the absorbance difference between light- and dark-adapted BR from time-dependent absorbance changes at 585 nm. At different temperatures the sample was illuminated ($\lambda > 500$ nm), and the absorbance changes (ΔA) induced by dark adaptation were monitored at 585 nm until spectral equilibration was reached. The absorbance changes were extrapolated back to time zero (i.e., termination of illumination). Since the reaction was found to be first order (see below), a valid extrapolation procedure could be used. The absorbance difference at 585 nm between the light-adapted state of BR (at $t = 0$) and the dark-adapted state (at $t = \infty$) can be easily and accurately determined in this way and is due to the difference in both the λ_{max} values and the extinction coefficients. This quantity represents a reliable measure of the extent of light adaptation. In Figure 4, the absorbance difference at a given temperature normalized to the value at 10 °C is plotted for BR both in the PM and in DMPC vesicles. The ordinate value "100%" represents 100% light adaptation, i.e., the chromophore retinal is exclusively in the all-trans configuration, whereas the "0%" ordinate value means 0% light adaptation, i.e., the dark equilibrium with the 1:1 mixture of both isomers. According to the data depicted in Figure 4, BR in the PM (●) undergoes complete light adaptation in the temperature range from 5 to 28 °C; at higher temperatures, the extent declines, down to a value of about 84% at 44 °C. The latter value corresponds to an isomeric ratio of 92% *all-trans*-BR to 8% 13-*cis*-BR in the sample. For other intrinsic properties of the PM, temperature-dependent changes around 23–31 °C have also been previously described; their origin, however, is unknown to date (Chignell & Chignell, 1975; Korenstein et al., 1976; Sherman & Caplan, 1978; Sherman, 1981; Hwang et al., 1981). In the temperature dependence of the extent of light adaptation for BR in the DMPC environment (○), more dramatic changes are observed. Between 1.3 and 10 °C, where BR is aggregated, 100% light adaptation occurs, but between 10 and 23 °C, the extent sharply declines to approximately 47% which corresponds to an isomer mixture of 73.5% *all-trans*- to 26.5% 13-*cis*-retinal. Whereas in the temperature region from 23

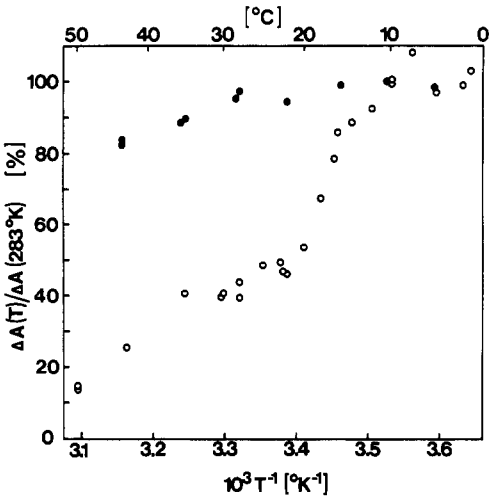

FIGURE 4: Temperature dependence of the extent of light adaptation for BR in DMPC vesicles (○, DMPC/BR = 94 and 106) and in the PM (●). Ordinate: absorbance difference at 585 nm between the light-adapted and the dark-adapted state of BR at a given temperature normalized to the value at 10 °C. An ordinate value of 100% represents 100% light adaptation, i.e., the chromophore retinal is exclusively in the all-trans configuration, whereas the 0% value represents 0% light adaptation, i.e., the dark equilibrium with the 1:1 mixture of *all-trans*- and 13-*cis*-retinal.

to 35 °C the extent remains relatively constant, it further decreases at higher temperatures with a slope slightly steeper than the one observed for the PM (Figure 4). At the highest temperature examined, i.e., 50 °C, nearly no light adaptation takes place (14.5% extent, which corresponds to 57% *all-trans*-retinal as chromophore in the BR molecules). When the flash photometric technique for determination of the isomer composition in the reconstituted system is applied, the values obtained are in good quantitative agreement with those from the method described previously. At 5.5 °C, both methods yield 100% *all-trans*-BR in the light-adapted state whereas at 30 °C only 72.5% according to the flash photometric technique or 70% according to the data of Figure 4 are *all-trans*-BR molecules. Comparison of the temperature dependencies of the extent of light adaptation (Figure 4) and the BR disaggregation/aggregation transition as determined by CD (Figure 3A) and by rotational diffusion measurements (Heyn et al., 1981a; Figure 4) reveals that both processes are strongly correlated in BR–DMPC vesicles. The sharp decrease in the extent of light adaptation occurs in the same temperature range as the reversible alteration in BR's aggregation state; furthermore, both transitions have their midpoints around 16–17 °C.

The dependence of the extent of light adaptation on BR's aggregation state could also be observed with BR reconstituted in SBPL and DPL vesicles. In these reconstituted systems, the lipid phase is always in the liquid-crystalline state in the investigated temperature range of 5–40 °C, and BR's aggregation state is determined by the lipid to BR ratio. Whereas in SBPL–BR vesicles of low lipid to BR ratio (49 mol/mol), in which according to the CD measurements BR is aggregated, a 10–11-nm difference in the absorption maxima and a 11% difference in the extinction coefficients of the light- and dark-adapted chromoprotein occur, the differences are only 5–6 nm and 0% for monomeric BR in vesicles with a molar SBPL to BR ratio of 262 at 10 °C. Similar features are observed in BR–DPL vesicles.

Rate of Dark Adaptation. The kinetics of dark equilibration of BR's chromophore from the light-adapted state were investigated by measuring the time dependence of the absorbance change at 585 nm, the wavelength of the maximal absorbance difference between the light- and dark-adapted states. Measurements performed at other wavelengths led to comparable results. At pH 5.0, the reaction rate is faster than at higher pH values, e.g., in the case of monomeric BR–SBPL vesicles about 15 times faster as compared to that at pH 6.9 (Table I), which allows the determination of the rates in reasonable times even at low temperatures. The increase in the rate constant of dark adaptation at acidic pH for BR in the PM is smaller (Table I).

With BR–DMPC vesicles, the kinetics of the dark adaptation reaction were measured between 1.3 and 50.0 °C. Over the whole temperature range, the reaction followed first-order kinetics. In the inset of Figure 5, the experimental data (circles) and the calculated fit with one exponential rate constant are shown at 43 °C. The Arrhenius plot of this rate constant (Figure 5 open and closed circles) exhibits a pronounced discontinuity in the temperature region between about 12 and 22 °C. The midpoint of this discontinuity and the one

Table I: Rate of Dark Adaptation of BR in Various Lipid Environments

sample	L/BR	temp (°C)	BR's state of aggregation	τ (min)
PM, pH 5.0[a]	7	5.0	aggregated	1049
		28.0	aggregated	30.4
PM, pH 6.9[b]		30.0	aggregated	48.2
BR-DMPC, pH 5.0[a]	106	5.0	aggregated	126.3
		28.0	monomeric	14.7
BR-DPPC, pH 5.0[a]	104	5.0	aggregated	137.5
BR-DPL, pH 5.0[a]	110	28.0	monomeric	1.8[c]
BR-SBPL, pH 5.0[a]	130	28.0	monomeric	2.2[c]
BR-SBPL, pH 6.9[b]	262	30.0	monomeric	30.0[c]
	49	30.0	aggregated	10.8[c]

[a] Sodium acetate buffer, 100 mM. [b] Phosphate buffer, 25 mM. [c] Kinetics are not single exponential; therefore, the time until the signal decayed to $1/e$ is given.

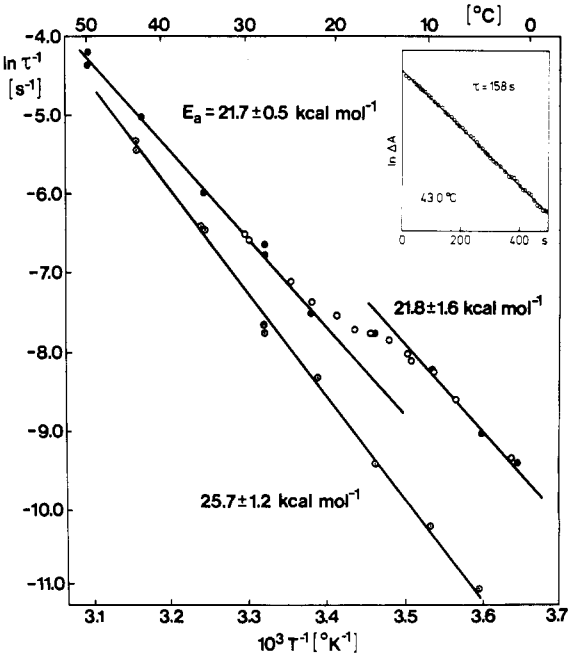

FIGURE 5: Arrhenius plot of the rate constant for dark adaptation in BR–DMPC vesicles (upper data set) and in the PM (lower data set); DMPC/BR values were 94 (○) and 106 (●). Straight lines are obtained by least-squares fits. E_a is the calculated activation energy. Inset: Kinetics of dark adaptation in BR–DMPC vesicles (DMPC/BR = 106) at 43 °C monitored as the absorbance change at 585 nm. Experimental data (○) and the calculated fit with one exponential rate constant (—) are shown.

Table II: Rate of Formation and Decay of M-410 in Various Lipid Environments

sample[a]	L/BR	temp (°C)	BR's state of aggregation	$\tau_{1/2}$,formation (ms)	$\tau_{1/2}$,decay[c] (ms)
PM	7	5.4	aggregated	0.250	38.7
		20.5	aggregated	0.054	7.3
		30.0	aggregated		3.0
BR-DMPC	94	5.5	aggregated	0.115[e]	213.0[f]
		21.0	monomeric[d]		49.3
		30.0	monomeric		23.5
BR-SBPL	130	5.0	monomeric		57.2
BR-DPL[b]	110	5.3	monomeric	0.038[e]	35.0[f]

[a] Experiments were carried out in 100 mM sodium acetate buffer, pH 5.0. [b] Experiment carried out in 150 mM KCl, pH 5.7. [c] Mean of two measurements. [d] Predominantly monomeric. [e] 5.1 °C. [f] Kinetics not single exponential.

found for the protein aggregation–disaggregation transition (Figure 3A) are both at around 16 °C. The experimental data in the temperature interval in which all BR molecules are either aggregated (1–10 °C) or monomeric (23–50 °C) lie on two nearly parallel straight lines which were obtained by least-squares fit. From their slopes, activation energies of 21.8 ± 1.6 kcal mol^{-1} (95% confidence interval) and 21.7 ± 0.5 kcal mol^{-1} for dark equilibration of aggregated BR and monomeric BR, respectively, have been calculated. As expressed in the discontinuity of the Arrhenius plot, however, the rate of this reaction is significantly different for both aggregation states. From the data extrapolated to equal temperatures, a 2.5 times smaller rate constant for monomeric BR was computed. The measurements were repeated with BR–SBPL vesicles containing aggregated and monomeric BR's (see above) which allow direct comparison at the same temperature and at the same physical state of the lipids (liquid crystalline). In this system, the dark adaptation rate of the BR monomers was found to be 2.8 times slower than the rate of the aggregates (Table I).

For comparison with the reconstituted system, the temperature dependence of the rate constant for dark adaptation of BR in the PM was determined under identical experimental conditions. The data shown in Figure 5 could be fitted with one straight line over the entire temperature range from 5.0 to 43.6 °C. The calculated activation energy of 25.7 ± 1.2 kcal mol^{-1} agrees fairly well with the values obtained for monomeric and aggregated BR in DMPC vesicles (Figure 5) as well as with recently reported values for native BR and with *all-trans*-retinal-regenerated bacterioopsin in the PM at different experimental conditions (Keen & Dencher, 1976; Tokunaga & Ebrey, 1978; Ohno et al., 1977). Figure 5 illustrates furthermore the influence of the lipid environment on the rate constant of this process. Dark adaptation proceeds faster in DMPC vesicles than in the PM; at 5 °C, for example, where BR in both systems forms a hexagonal protein lattice with identical unit cell dimensions, the reaction rate is 8.3 times higher in the DMPC environment.

Table I summarizes the kinetic data of dark adaptation for BR in different lipid environments at pH 5.0 and 6.9. Aggregated BR's in DMPC and DPPC have very similar time constants. The rates of monomeric BR dark adaptation in SBPL and DPL are also very similar but 6.7 and 8.1, respectively, times faster than that for monomeric BR in DMPC. When the differences in the aggregation state, temperature, and pH value of the various samples listed in Table I are considered, and the effect of these parameters on the reaction rate, BR undergoes a 4.5–50 times faster dark adaptation in SBPL and DPL as compared to in the PM.

Photocycle Kinetics. Upon absorption of a photon, BR undergoes a complex photoreaction cycle expressed by characteristic absorbance changes in the visible and ultraviolet

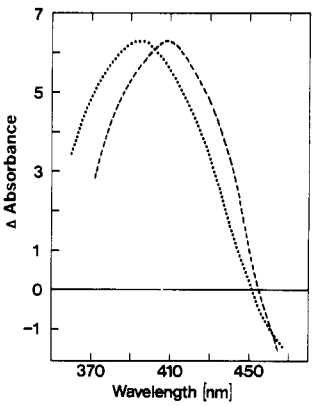

FIGURE 6: Light-induced difference spectrum of the photocycle intermediate M-410 in BR–DMPC vesicles at 4 (- - -) and 26 °C (···), respectively. DMPC/BR = 94. Ordinate: absorbance difference (arbitrary units) between illuminated and nonilluminated samples. Curves are normalized to identical peak heights.

spectral range [for recent review, compare Stoeckenius et al. (1979), Ottolenghi (1980), and Birge (1981)]. The molecular changes accompanying the photocycle of *all-trans*-BR are responsible for the vectorial proton translocation by this photopigment. Any differences in the photocycle of aggregated and monomeric BR might thus give a hint concerning the molecular mechanism of this light-driven proton pump.

Flash-induced transmission changes monitored at a variety of wavelengths confirmed the formation and decay of the photocycle intermediates K (^{610}T), L (^{550}T), and M (^{411}T) for both aggregated and monomeric BR in DMPC vesicles. Spectral changes characteristic of the intermediate O (^{646}T), however, were not observed in BR–DMPC and BR–DMPC/PS (95/5 w/w) vesicles for both aggregation states, i.e., at 5.0 and 31.3 °C. The decay rate of the intermediate M-410 was investigated in more detail. Since the lifetime of M-410 is 5.5–7.8 times longer in DMPC vesicles in comparison with the PM under identical conditions (Table II), measurable concentrations of this intermediate can be accumulated even during illumination with the relatively small light intensity applied. In Figure 6, the absorbance spectra of BR–DMPC vesicles under continuous illumination are plotted for two different temperatures. The spectrum obtained for aggregated BR at 4 °C has its maximum at 410 nm and an isosbestic point at 455 nm. These values are characteristic for the intermediate M-410 in the photocycle of *all-trans*-BR. Very similar difference spectra were obtained by flash spectroscopy of BR in the PM (Dencher & Wilms, 1975; Kung et al., 1975; Lozier et al., 1975). At 26 °C, where BR is in the monomeric state (Figure 3A), a slightly blue shifted spectrum of similar band shape is observed.

Flash-induced time-dependent transmission changes at 410.5 nm were measured in BR–DMPC vesicles over the tempera-

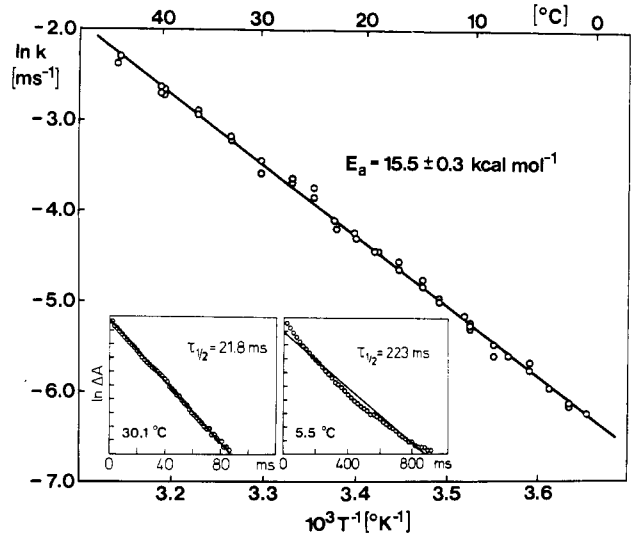

FIGURE 7: Arrhenius plot of the rate constant for the decay of the photocycle intermediate M-410 in 100 mM sodium acetate buffer, pH 5.0. DMPC/BR = 94. The straight line is obtained by a least-squares fit. E_a is the calculated activation energy. Insets: Flash-induced (λ = 580 nm) absorbance changes at 410.5 nm plotted logarithmically against time (O) and calculated fit with one exponential rate constant (—).

ture range of 0.6–45 °C. At every temperature selected, two to four measurements were performed. The kinetic data calculated from the transmission changes were found to be quite reproducible (compare Figure 7; e.g., at 40.3 °C, $\tau_{1/2}$ = 10.2 ± 0.4 ms, mean value and standard deviation of four measurements). Since transmission changes at 410.5 nm were observed over the whole temperature range from 0.6 to 45 °C, the photochemical cycle apparently operates for both aggregated and monomeric BR. In the insets of Figure 7 the absorbance changes are plotted logarithmically against time. Whereas at 30.1 °C the decay can be accurately described by a single exponential, at 5.5 °C clear deviations occur, and a satisfactory fit of the data could only be obtained with two exponentials differing by a factor of 1.7 in their rate constants. In the temperature range between 45 and about 13 °C, the decay was found to be single exponential; the introduction of a second exponential did not lead to any improvement in the fit. Below 13 °C, however, more or less pronounced deviations from a single first-order process were apparent, and a satisfactory description of the data was possible only with two exponentials. It should be mentioned that the curvature of the experimental data shown in the inset at 5.5 °C represents the most pronounced deviation from a single exponential decay observed. For comparison of the data obtained over the entire temperature range, all decay kinetics were fitted with a single exponential, and the calculated rate constants were presented in the form of an Arrhenius plot (Figure 7). This is meaningful since the temperature-dependent alteration in the reaction order of the decay process is not correlated with the changes in both the physical state of the lipids and the aggregation state of BR (see Discussion). In the Arrhenius plot (Figure 7), one straight line obtained by a least-squares approximation does reasonably well connect all data points over the entire temperature range investigated. The calculated activation energy of 15.5 ± 0.3 kcal mol^{-1} (95% confidence interval) is similar to previously reported values for the decay of M-410 in the PM (Dencher & Wilms, 1975; Kung et al., 1975; Packer et al., 1981).

For aggregated BR in DMPC vesicles, the rate of M-410 formation is about twice as fast as in the PM, and its decay in 5.5 times slower (Table II). The formation of this intermediate is even more accelerated for monomeric BR in SBPL (data not shown) and DPL (Table II) although in these lipid environments the decay rates are less affected as in DMPC and resemble those in the PM.

Discussion

The experiments described in this report were designed to compare in a quantitative way the photocycle and the light–dark adaptation reactions of aggregated and monomeric bacteriorhodopsins with the aim of defining the smallest functional unit of this membrane protein. The size of the functional unit could be different for these different processes. Knowledge of the functional unit is required for the construction of any working model of the system, and any differences observed in the efficiency of monomeric and aggregated BR will help to understand this proton pump at the molecular level. Furthermore, since BR was reconstituted into different lipid environments, i.e., different with respect to the chemical nature and physical state of the lipids, the effect of protein–lipid interactions on BR's function could be examined.

Recently it was demonstrated that monomeric BR is able to translocate protons (Dencher & Heyn, 1979) and even to generate a steady-state electrochemical potential difference for protons of the same magnitude as hexagonally aggregated BR (Casadio & Dencher, 1981). Although these experimental approaches yielded fundamental information for the localization of the basic structural unit of H^+ translocation, they did not measure the true efficiency of the system. Furthermore, this approach is handicapped by the finding that the degree of coupling between the photoreaction cycle and proton translocation is variable and depends on experimental conditions. The ionic strength (Ort & Parson, 1979; Bogomolni et al., 1980; Govindjee et al., 1980; Kuschmitz & Hess, 1981) and the pH of the external medium (Renard & Delmelle, 1980) modulate the number or protons pumped per photon absorbed by BR without any observable alteration in the photoreaction cycle. Similar results were obtained from studies in which the C-terminal peptide chain of BR had been removed by enzymatic digestion (Govindjee et al., 1982). Therefore, the investigation of spectroscopic changes induced by the photocycle, which drives proton translocation across the membrane, and by the light–dark adaptation reaction, which determines the isomeric composition of the chromophore, will yield knowledge about elementary steps in BR function and their dependence on BR's aggregation state and on the lipid environment.

Effect of BR's Aggregation State and the Lipid Phase on the Photoreaction Cycle. Comparison of BR in different vesicle systems with BR in the PM reveals that the *all-trans*-BR photocycle is not influenced much by protein–protein interactions. Spectral changes characteristic for the appearance and decay of the intermediates ^{610}T (K), ^{550}T (L), and ^{411}T (M) in the native membrane are also observed for both aggregated and monomeric BR in the reconstituted systems. Unrelated to the aggregation state, however, the intermediate ^{646}T (O) was not detected in DMPC and DMPC–phosphatidylserine (95:5 w/w) vesicles containing BR. In fact, this was the only parameter observed to be perturbed by the reconstitution procedure itself. This is in accordance with previous reports claiming that the occurrence of the ^{646}T intermediate is very dependent on the experimental conditions (Dencher & Wilms, 1975; Sherman et al., 1976; Dencher & Heyn, 1978). Hexagonally aggregated BR in DMPC vesicles exhibits the same ground-state absorption spectrum as BR in the PM (Figure 1); also, the absorption spectrum of the in-

termediate M-410 (Figure 6) and the activation energy for its decay (E_a = 15.5 ± 0.3 kcal mol^{-1}, Figure 7) are very similar. This indicates that neither the reconstitution procedure nor the chemical nature and the physical state of the lipids influence these parameters. This contrasts with the report of Sherman & Caplan (1978) of a drastically decreased activation energy for M-410 decay in the PM in which the native lipids have been replaced by DPPC and egg lecithin. BR reconstituted with DMPC by means of a freeze–thaw sonication technique shows at 10 °C (where the protein is aggregated) for the light- and dark-adapted state a λ_{max} of 560 and 551 nm, respectively, and a $\Delta\epsilon$ of about only 5% (Casadio & Stoeckenius, 1980) whereas the respective values in the BR–DMPC vesicles obtained by the detergent dialysis technique are 566 and 556 nm (Figure 2) and 13% (Figure 1). The latter values are very close to the values known for BR in the PM (Oesterhelt et al., 1973).

The intermediate M-410 was investigated in detail to compare aggregated and monomeric BR on a more quantitative level. The investigation of the decay kinetics of M-410 seems to be especially suitable for this purpose because this reaction step is assumed to be a "key step" in the proton translocation mechanism. Furthermore, the decay rate has been found to be affected in a quite sensitive way by alterations in BR's microenvironment (Oesterhelt & Hess, 1973; Dencher & Wilms, 1975; Korenstein & Hess, 1977; Yoshida et al., 1977). Figure 7 shows that the logarithm of the rate constant for the decay of M-410 is a linear function of the inverse temperature over the entire temperature range investigated. This means that when the rate constant for monomeric BR is compared with that for aggregated BR at the same temperature by extrapolation along the Arrhenius plot, identical rate constants are obtained. The Arrhenius plot demonstrates that neither the changes in BR's aggregation state around 16 °C nor the lipid phase transition around 23 °C (Figure 3) influences the decay kinetics. These transitions do not seem to be the cause for the observed change in the description of the reaction kinetics from one to two exponentials at temperatures below 13 °C since they occur at higher temperatures. We have checked the possibility that photoselection effects by the polarized laser excitation flash are not a reason for the second component in the decay kinetics. Control experiments with the PM under identical conditions showed a similar temperature dependence on the reaction kinetics (data not shown). In numerous reports (Kuschmitz & Hess, 1981; Ohno et al., 1981; Mäntele et al., 1981), it was stated that one or two exponentials are necessary for a good description of the M-410 decay, depending on such experimental parameters as ionic strength, pH, and excitation intensity. It is worth mentioning that any uncertainty in the data evaluation at temperatures below 13 °C does not affect our statement that the decay kinetics of M-410 are not affected by BR's state of aggregation since at 13 °C most of the BR molecules are aggregated (Figure 3) and their kinetics can be compared with those of the BR monomers at higher temperatures. Furthermore, these results obtained with BR–DMPC vesicles are in accordance with preliminary measurements with BR–SBPL vesicles which have the advantage that monomeric and aggregated BR can be compared at the same temperature and the same physical state of the lipids. The aggregation state of BR in this lipid depends on the lipid to protein ratio. The rate constants for M-410 decay of aggregated (L/BR = 49) and monomeric (L/BR = 262) BR were determined at equal temperatures between 5 and 20 °C and found to be quite similar. Description of the resulting transmission changes with either one or two exponential rate constants reveals that in this system (25 mM phosphate buffer, pH 6.9) the decay of M-410 is only slightly faster (≤16%) for aggregated BR (N. A. Dencher, unpublished results).

It could also be argued that the native lipids of the PM which had not been removed prior to reconstitution of BR might always surround this membrane protein as a boundary layer and thereby prevent any alterations of the photocycle kinetics induced by the lipid phase transition of DMPC. This argument can be excluded for two reasons. On the one hand, due to the low lipid to BR ratio of the PM, the number of native lipids is not large enough to cover the surface of the membrane proteins completely when they disaggregate to the state of monomers (Heyn et al., 1981b). Therefore, at least in the case of BR dimers and monomers, part of their hydrophobic surface has to be in contact with the foreign lipid species and can sense the lipid phase transition. On the other hand, the chemical nature of the lipids influences the rate of the photochemical cycle (Table II) and also of the light–dark adaptation reaction (Table I). This occurs for both aggregation states; therefore, even the BR trimer is not protected by the native lipids against the action of the foreign lipid phase. The investigated decay rate of M-410 is affected by the chemical nature but not by the physical state of the lipid phase.

In the reconstituted BR–DMPC vesicles as well as in the other reconstituted systems, monomeric BR undergoes both fast rotational diffusion around the membrane normal (e.g., 15-μs rotational relaxation time at 30 °C; Heyn et al., 1981a) and fast translational motion in the membrane plane (e.g., translational diffusion coefficient of 1.8 μm^2 s^{-1} at L/BR = 140 and 28.5 °C; Peters & Cherry, 1982) at temperatures above the lipid phase transition. It might be speculated that short-lived transient aggregates formed by collisions of monomeric BR during or immediately after the actinic flash are responsible for the transmission changes of the investigated M-410 decay [as well as for the observed proton pumping capability (Dencher & Heyn, 1979)] of the BR monomers. Since in the hexagonally aggregated state induced either by the lipid phase transition or by the low lipid to BR ratio all BR molecules are in "permanent collision", contrary to the very minor number of short-lived aggregates generated by transient "dynamic collision" of BR monomers in the liquid-crystalline lipid phase at high lipid to BR ratios, large differences in the signal amplitude (as well as the number of translocated protons) should occur. This and the expected dependence on the lipid to BR ratio, which determines the probability of collisions, were not observed in our experiments. If aggregated and monomeric BR's would have different rate constants for M-410 decay, then the monitored transmission changes of BR in the monomeric state should be composed of a sum of two exponentials representing the kinetics of excited monomers and of excited transient short-lived aggregates, whereas in the hexagonal lattice all BR molecules should behave equally. In the case of BR monomers in DMPC vesicles, however, the transmission changes representing the decay of the intermediate M-410 were found to be single exponential.

Weak excitation intensities were applied which excited less than about 7% of all BR molecules in the sample per flash. This was done not only for the prevention of multiple photon hits but also mainly for the following two reasons: (1) Previous data of the dependence of the photocycle kinetics on the excitation intensity had been explained by the assumption of kinetic cooperativity in the decay of M-410 in the PM (Korenstein et al., 1979; Ohno et al., 1981). In the trimeric array of BR, *excited* BR molecules are supposed to influence the

decay kinetics of their neighbors. The aim of the present investigation was to determine the smallest structural unit which is still able to perform the basic functions of BR; therefore, this kind of interaction should be prevented since in the case of monomeric BR it might obviously be absent. (2) Recent investigations demonstrated that the kinetics of BR's photochemical cycle are influenced by the electrochemical protonmotive force across the membrane protein (Hellingwerf et al., 1978; Quintanilha, 1980). The alterations in the kinetics can be substantial. The half-time of M-410 decay increases 4–5-fold as the membrane potential increases from 0 to 100 mV (Helgerson et al., 1982). Upon a series of flash excitations with a repetition rate higher than the decay rate of the electrochemical gradient, a very large electrochemical gradient might develop. The magnitude of the electrochemical gradient generated and the degree of perturbation of the photocycle kinetics depend on the permeability properties of the membrane systems. Since the permeability is determined by the physical state of the lipid phase, this effect may even be the source of breaks or discontinuities in an Arrhenius plot near the lipid phase transition temperature. Therefore, in our experiments, data were only collected upon single flash excitation. Repetitive excitations of the same sample were only applied after time intervals larger than the relaxation time of the electrochemical gradient (e.g., 21 min for BR–DMPC vesicles at 10 °C). However, since the electrical membrane potential is built up very rapidly [values of 80 μs for intact *Halobacterium halobium* cells (Szalontai, 1981) and about 20 μs for BR–SBPL vesicles (Johnson et al., 1981) have been determined], even upon single flash excitation the photocycle rates might be perturbed. So that this perturbation could be minimized, the size of the generated electrochemical proton gradient was kept as low as possible in our experiments by exciting only less than 7% of the BR molecules in the investigated vesicle systems.

Although the data presented demonstrate that the decay kinetics of the intermediate M-410 are not affected either by the physical state of the lipid phase or by the aggregation state of the chromoprotein, other steps in the *all-trans*-BR and 13-*cis*-BR photocycle might be influenced by these parameters. This possibility remains to be investigated by means of experiments like those described here. Photoelectrical measurements on BR–DMPC/PS vesicles attached to planar lipid bilayers show that the rates of flash-induced photocurrents which are correlated with the formation of M-410 during the photocycle are dependent on BR's state of aggregation. This phase of the photocurrent is about 3 times faster for monomeric BR as compared to that for the aggregated pigment (A. Fahr and N. A. Dencher, unpublished results). Furthermore, the formation of M-410 for Triton X-100 solubilized monomeric BR is about 3 times faster than for BR in the PM in detergent-free buffer solution, whereas the half-life of the decay is the same in both systems (Dencher & Heyn, 1978).

Whereas the decay rate of M-410 is influenced neither by the state of aggregation of BR nor by the physical state of the lipid phase, the chemical nature of the lipids does affect this process (Table II). The slow down of the decay rate is more pronounced in the case of the saturated lipid DMPC than for DPL with its branched hydrocarbon chains composed of isoprene units (as the native lipids of halobacteria) and the unsaturated SBPL. In BR–DMPC vesicles, the M-410 decay rate is decreased 5.5–7.8 times as compared to the rate in the PM, independent of BR's state of aggregation (Table II).

Effect of BR's Aggregation State and the Lipid Phase on the Extent and Rate of Light–Dark Adaptation. The C-13 double bond of the retinylidene moiety of BR has the alternative of being either in the trans or in the cis configuration. After dark equilibration, the PM contains a mixture of *all-trans*-BR and 13-*cis*-BR in a ratio of about 1:1 (Dencher et al., 1976a,b; Sperling et al., 1977; Maeda et al., 1977; Pettei et al., 1977; Ohno et al., 1977). Upon light adaptation with moderate light intensities, only *all-trans*-BR is present (Oesterhelt et al., 1973; Sperling et al., 1979). Whereas retinal isomers in solution are fairly stable and do not isomerize to any extent at room temperature without catalyzer (Sperling et al., 1977), the BR isomers undergo fast equilibration in the time range of minutes to hours (Figure 5). This indicates that the protein moiety of BR, BO, functions as a catalyzer, an isomerase.

A first indication of an effect of BR's state of aggregation on the light–dark adaptation reaction arose from experiments performed with solubilized BR (Dencher & Heyn, 1978; Casadio et al., 1980) and with BR–DMPC vesicles (Cherry et al., 1978; Casadio & Stoeckenius, 1980). In accordance with these previous investigations, the present measurements show that instead of the 10-nm band shift of the visible absorption spectrum during light–dark adaptation, characteristic for hexagonally aggregated BR, a shift of only 5 nm or even less occurs for monomeric BR in DMPC vesicles. In addition, the absorbance difference decreases (Figures 1 and 2). These changes are not due to an altered isomer composition of the chromophore retinal of dark-adapted BR since there is only a small shift of the absorption maximum of the dark-adapted BR–DMPC sample in the temperature range of the protein disaggregation transition (Figure 2). These data are in accordance with the measured isomeric composition of the chromophore retinal in dark-adapted monomeric and aggregated BR on the basis of the flash spectroscopic technique (see Results) as well as direct extraction of the chromophore from a BR–DMPC vesicle system similar to the one used in the present investigation (Casadio & Stoeckenius, 1980). As indicated by the pronounced temperature dependence in the wavelength of the absorption maximum of light-adapted BR in DMPC vesicles (Figure 2), it is the extent of light adaptation, i.e., the percentage of 13-*cis*-BR isomerized to *all-trans*-BR upon illumination with moderate light intensities, which differs for aggregated and monomeric BR. From the data of Figure 4, it can be read off that light-adapted aggregated BR contains exclusively *all-trans*-retinal as chromophore whereas light-adapted BR monomers at 23 °C contain about 27% of the 13-cis isomer. This corresponds to a 53% decrease in the extent of light adaptation as compared to aggregated BR. A similar value for the extent of light adaptation of monomeric BR in DMPC vesicles reconstituted by means of a freeze–thaw sonication technique has been previously reported by Casadio & Stoeckenius (1980).

The change in the isomer composition of light-adapted BR is caused by changes in the protein–protein interactions (aggregated vs. monomeric BR) and is not a result of changes in the protein–lipid interactions. The shape of the temperature dependency of the extent of light adaptation (Figures 2 and 4) in BR–DMPC vesicles parallels the crystallization–disaggregation transition of the protein molecules as monitored by CD (Figure 3A) and by flash-induced transient linear dichroism measurements (Heyn et al., 1981a) but not the lipid phase transition (Figure 3B). Futhermore, comparison of the extent of light adaptation in BR-SBPL vesicles performed at the same temperature and the same physical state of the lipid

phase (liquid crystalline) yields a similar decrease in the extent for monomeric BR.

It can be ruled out that the dark adaptation reaction investigated in this report accounts for the large amount of 13-*cis*-BR in the light-adapted sample of monomeric BR since its rate is too slow (Figure 5 and Table I) to generate any appreciable concentration of this isomeric species during the few seconds between termination of illumination and determination of the isomer composition. To account for the observed decrease in the extent of light adaptation in monomeric BR, the existence of a second reaction pathway from *all-trans*-BR to 13-*cis*-BR has to be postulated whose efficiency is regulated by the aggregation state of BR. This could be either a dark reaction from *all-trans*-BR itself or one of its photocycle intermediates to 13-*cis*-BR or its intermediates as previously discussed by Casadio et al. (1980) or a photoreaction from the *all-trans*- to the 13-*cis*-BR system similar to the one observed for *all-trans*-BR in the PM under high light intensities (Sperling et al., 1979). Another possibility is that in the monomeric state a proportion of the 13-*cis*-BR molecules in the dark equilibrium mixture does not undergo photoisomerization to *all-trans*-BR. In aggregated BR, the intermediate ^{610}C of the 13-*cis*-BR photocycle decays to both 13-*cis*-BR and *all-trans*-BR. The majority of the ^{610}C molecules return to 13-*cis*-BR (Dencher et al., 1976a; Sperling et al., 1977, 1979). In the case of monomeric BR, the pathway from ^{610}C to *all-trans*-BR could be much less probable or even be blocked for a fraction of the molecules. Further experiments are necessary to distinguish between these three possibilities.

Not only the extent of light adaptation (Figure 4) but also the rate of dark equilibration from the light-adapted state exhibits a marked temperature dependency in BR–DMPC vesicles (Figure 5). The nonlinear shape of the Arrhenius plot of the rate constant for this process (Figure 5) resembles the temperature dependence of the extent of light adaptation (Figure 4) as well as of the CD transition curve (Figure 3) and of BR's rotational correlation time (Heyn et al., 1981a). The transition curve of all four processes exhibit a midpoint of the respective transitions around 16–17 °C and plateau values at temperatures below 10 °C and above 23 °C. Therefore, it is BR's aggregation state that influences the rate of dark equilibration of the chromophore retinal in this photopigment. In the transition region, the reaction rate seems to be nearly temperature independent, exhibiting a rather small activation energy. Although aggregated and monomeric BR's have the same activation energies for dark adaptation, which are similar to the one determined for BR in the PM under identical experimental conditions (Figure 5), the respective rates are significantly different. From the data of Figure 5, it can be calculated that the rate constant of monomeric BR is about 2.5 times smaller than the one of the aggregated chromoprotein. The triphasic form of the Arrhenius plot cannot be explained as a break around 23 °C resulting from the gel to liquid-crystalline lipid phase transition since also in BR–SBPL vesicles at the same temperature and physical state of the lipid phase the dark adaptation rate of the BR monomers is about 3 times slower than the rate of the aggregated BR molecules (Table I). An intrinsic temperature dependency of BR itself as the reason for the breaks in the Arrhenius plot can be excluded by the observation that for BR in the PM the respective plot is linear over the entire temperature range investigated (Figure 5). The observed decrease in the rate of dark adaptation of the monomer has to be explained by an altered conformational state of the protein moiety influenced by the reversible disaggregation of the lattice. If the change in the reaction rate would have been caused by changes in the protein–lipid interactions, e.g., because in the case of the monomer a much larger proportion of its hydrophobic surface is in contact with the artificial lipid environment as compared to the aggregates, the rate should be faster instead of slower. The data of Figure 5 and Table I clearly show that in all artificial lipid environments investigated, BR's rate of dark adaptation is faster than or the same as that in the PM. The physical state of the lipid environment, however, has no influence on this process, a phenomenon also observed for the photocycle kinetics. Our observation that BR's state of aggregation determines its rate of dark adaptation and the idea that differences in the conformational state of BR in the presence and absence of interacting neighbors are the reason for this behavior are in accordance with previous results of other obtained with different systems (Ebrey et al., 1977; Peters & Peters, 1978; Papadopoulus & Cassim, 1979). Although the enzymatic activity of monomeric BR is less than that for aggregated BR in the same environment as expressed by the slower reaction rate, the underlying molecular mechanism seems to be the same. The isomerase has not only the same activation energy (Figure 5) but also the same stereospecificity in the two aggregation states since in the monomeric state also only *all-trans*- and 13-*cis*-retinal were extracted (Casadio & Stoeckenius, 1980). It should be remembered that the changes in the reaction rate induced by the altered chemical nature of the lipid environment are more pronounced than the one induced by the altered aggregation state of BR.

Conclusions. The results of this investigation, that the photoreaction cycle of *all-trans*-BR is basically the same for monomeric and aggregated BR and that no differences occur in the decay kinetics of the cycle intermediate M-410, are in accordance with the observation of monomeric BR being an efficient light-driven proton pump (Dencher & Heyn, 1979; Casadio & Dencher, 1981). Furthermore, comparable photoelectrical signals are induced by BR in both aggregation states (Bamberg et al., 1981; Rayfield, 1982). Therefore, the BR monomer seems to be the functional unit for proton translocation, and any model to explain the molecular mechanism of this light-driven proton pump has to be based on the monomer itself.

The light-induced bleaching of the chromophore in the presence of hydroxylamine as well as the regeneration of bacterioopsin with retinal are also affected by BR's state of aggregation; e.g., regeneration proceeds slightly slower in the monomer as in aggregated BR (N. A. Dencher, unpublished results). This is in accordance with the recently reported cooperativity of these processes in the PM (Becher & Cassim, 1977; Rehorek & Heyn, 1979).

Only the *all-trans*-BR molecule has been shown to be able to pump protons (Ohno et al., 1977; Lozier et al., 1978; Fahr & Bamberg, 1982). Since light-adapted monomers contain only 70% *all-trans*-retinal, we expect that a sample of monomeric BR is about 30% less active as a light-driven proton pump than the corresponding sample of aggregated BR. A further advantage of the organization of the BR molecules as aggregates is the higher stability against harmful environmental factors such as elevated temperature and extremes of pH.

In conclusion, it might be stated that for all the various reactions of *all-trans*-BR investigated so far, the monomer is functional. For some of these processes, e.g., those involved in light–dark adaptation, BR–BR interactions regulate the efficiency of the respective reaction steps.

Registry No. DMPC, 13699-48-4; DPPC, 2644-64-6; DPL, 32448-32-1.

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Calcium-Sensitive Binding of Heavy Meromyosin to Regulated Actin Requires Light Chain 2 and the Head–Tail Junction†

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ABSTRACT: Sedimentation in a preparative ultracentrifuge was used to determine the affinity of heavy meromyosin, HMM, for regulated actin, F-actin plus troponin–tropomyosin, in the presence of MgATP at pH 7.0, 20 °C, and μ = 18 mM. HMM was prepared from vertebrate striated muscle myosin by a mild chymotryptic digestion. This HMM contained 85–90% intact 19 000-dalton light chains, LC2. In the presence of calcium, 90% of the HMM bound to regulated actin with an association constant of (2–4) × 10^4 M^{-1}. In the absence of calcium, while one-third of the HMM bound with an affinity similar to that observed in the presence of calcium, the rest bound much more weakly. It was not possible to accurately determine the association constant for this weakly binding HMM, but a 20-fold reduction in affinity is consistent with the binding data. The binding of single-headed heavy meromyosin to regulated actin was similarly sensitive to the calcium concentration. Since removal of calcium inhibits the regulated actin-activated ATPase of HMM greater than 20-fold, troponin–tropomyosin must be capable of inhibiting both the binding of HMM to regulated actin and a step which occurs after binding but prior to product release. Removal of LC2 increased the fraction of HMM with calcium-insensitive binding, and adding LC2 back to this depleted HMM restored most of the calcium sensitivity. Chymotryptic cleavage of LC2 to a 17 000-dalton fragment destroyed the calcium-sensitive binding of HMM to regulated actin. Phosphorylation of LC2, however, had no detectable effect on this binding. Thus, the calcium-sensitive binding of HMM to regulated actin requires that both the head–tail junction and the N-terminal part of LC2 be intact. Binding studies with cross-linked regulated actins and kinetic measurements of the rates of change in turbidity demonstrate that this calcium sensitivity is due to calcium binding to troponin and not to LC2.

In vertebrate striated muscles, contraction is regulated by calcium binding to troponin–tropomyosin on the thin filament (Ebashi et al., 1969). In the absence of calcium, troponin–tropomyosin inhibits the cyclic interaction of actin with the myosin cross bridges and, hence, force development. Calcium binding to troponin causes a change in the position of tropomyosin on the thin filament (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973), allowing for the myosin cross bridge to interact with actin and force to be developed. It has been thought that relaxed muscle has low stiffness because tropomyosin in the absence of calcium prevents myosin binding to actin either by physically blocking the myosin binding sites on actin (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973) or by causing a conformational change in the actin. Differences in X-ray diffraction patterns of relaxed and contracting muscles (Huxley, 1972) and the random orientations of spin-labels (Thomas & Cooke, 1980) and fluorescent dyes (Borejdo & Putnam, 1977) attached to the cross bridges in relaxed glycerinated fibers also indicate that most, if not all, of the myosin cross bridges are dissociated from actin during relaxation. However, recent in vitro binding studies suggest that a significant fraction of the cross bridges may be attached to the thin filament even during relaxation (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982; Inoue & Tonomura, 1982).

Regulated actin, F-actin plus troponin–tropomyosin, is an in vitro model of the thin filament. It activates the MgATPase of myosin in the presence but not in the absence of calcium (Weber & Murray, 1973). Similar calcium sensitivities are found for the regulated actin-activated ATPases of heavy meromyosin (HMM),[1] a two-headed proteolytic fragment of

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