# Effect of Protein–Protein Interaction on Light Adaptation of Bacteriorhodopsin†

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ABSTRACT: Triton X-100 solubilized monomers of bacteriorhodopsin (bR) show a decrease in the extent of light adaptation; the red shift and the absorbance increase of the visible absorption band are reduced to less than half the values observed in purple membrane (p.m.) with a corresponding reduction in the isomerization of 13-*cis*- to *all-trans*-retinal. Cross-linking of bR with glutaraldehyde before exposure to Triton prevents dissociation of the lattice and reduction in light adaptation. Experiments with cross-linked and lipid-extracted p.m. show that Triton effectively substitutes for the native membrane lipids and that the lattice structure apparently stabilizes the light-adapted state of bR under illumination. In lipid vesicles at molar lipid protein ratios ≥ 80, bR exists as monomers above the lipid-phase transition and aggregates below the phase transition. Above the lipid-phase transition light adaptation in the monomers, measured as either the red shift of the visible absorbance maximum or the isomerization of 13-*cis*- to *all-trans*-retinal, is also reduced to less than half of the extent observed in intact purple membrane or in the bR aggregates formed in lipid vesicles below the phase transition. At very high lipid to protein ratios, bR molecules cannot aggregate when the temperature is decreased below the phase transition, and these monomers in a solid lipid phase show the same reduced extent of light adaptation as monomers above the phase transition, thus confirming that this effect is mainly due to the absence of protein–protein interaction and not to the state of the lipid. The extent of the red shift upon light adaptation may be used as a convenient indicator to distinguish the aggregated and monomeric states of bR.

The purple membrane (p.m.) of halobacteria is a light-driven proton pump which is embedded as patches in the plasma membrane of these cells. Its pigment, bacteriorhodopsin (bR), is a retinal protein, which closely resembles the visual pigment of animals. In intact cells, bR forms a two-dimensional hexagonal lattice constituting 75% of the mass of the purple membrane patches; the remainder is lipid. The chromophores show a strong positive circular dichroic (CD) band and through exciton interaction within the lattice also a superimposed positive–negative exciton band, which disappears when the lattice dissociates. The negative CD band centered at ~600 nm has been used as an indicator for the presence of the lattice structure. For reference and further details of the structure and function of the purple membrane see Stoeckenius et al. (1979).

The purple membrane exists in two forms, light adapted (LA) and dark adapted (DA), with absorbance maxima ($\lambda_{max}$) at 568 and 558 nm, respectively. The light-adapted form which has a 12% higher absorbance is generated by exposure of dark-adapted p.m. to moderate light intensities for a few seconds to a few minutes. In the dark it slowly returns to the dark-adapted form with half-times which are strongly dependent on temperature and pH but typically are orders of magnitude larger than the half-times for light adaptation. It has been convincingly demonstrated that dark-adapted p.m. contains two species of bR in equal amounts; one, cbR, yields 13-*cis*-retinal upon extraction, and the other, tbR, yields *all-trans*-retinal (Sperling et al., 1977; Ohno et al., 1977; Oesterhelt et al., 1973; Pettei et al., 1977). The bR in light-adapted p.m. contains mainly or exclusively *all-trans*-retinal (Sperling et al., 1977).

Treatment with Triton X-100 dissociates p.m. into monomers of bR (Reynolds & Stoeckenius, 1977), and we have shown that upon illumination the increase in absorbance and extent of the red shift are reduced and increased amounts of 13-*cis*-retinal are found in fully light-adapted preparations (Casadio et al., 1980). Both cbR and tbR undergo cyclic photoreactions. Light adaptation occurs because one of the cbR photoreaction-cycle intermediates decays into two different thermal products; one is the next intermediate of the cbR cycle, and the other crosses over into the tbR cycle (Sperling et al., 1977; Ohno et al., 1977; Lozier et al., 1978). A back-reaction from the all-trans to the 13-cis cycle accounts for the decreased extent of light adaptation in Triton-solubilized p.m. (Casadio et al., 1980). In Triton-solubilized monomers it was not possible to determine whether the modification in the mechanism of light adaption is due to the change in lipid environment of the protein or to dissociation of the lattice. The results presented here indicate that the existence of the lattice is required for full light adaptation and that the requirements for a lipid environment are rather unspecific.

## Materials and Methods

*Materials.* Growth of *Halobacterium halobium* $R_1$ and isolation of purple membrane have been described (Oesterhelt & Stoeckenius, 1974). Soybean lecithin, egg lecithin, dimyristoyllecithin (DMPC), dipalmitoyllecithin (DPPC), and azolecithin were purchased from Avanti Biochemicals Inc., Calbiochem, and Sigma Chemical Co., pure glutaraldehyde (10% solution) was from Electron Microscopy Sciences, and Triton X-100 was from Sigma Chemical Co. All reagents were used without further purification. Deoxycholic acid (DOC) (Sigma Chemical Co.) was recrystallized as described (Hwang & Stoeckenius, 1977).

*Spectroscopy and Analytical Techniques.* Absorbance spectra were recorded with a Cary 14 spectrophotometer and a Nicolet 1180 computer as described (Casadio et al., 1980). Suspensions of liposomes prepared under the same conditions

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as the purple membrane vesicles or the hydroxylamine-bleached, bR-containing vesicles (Cherry et al., 1978) were used as reference samples. The absorbance maxima are reproducible with an accuracy of ±1.0 nm. CD spectra were recorded with a Jasco ORD/UV5 spectrophotometer with a 1-cm quartz cuvette in a thermostated holder.

The bR concentrations in purple membrane suspensions were determined from the absorbance at 560 nm by assuming a molar extinction coefficient for $bR^{DA}$ of 51 000 (R. Bogomolni, personal communication). Phosphorus was determined as described (Bartlett, 1959). The lipid to protein ratios in vesicle preparations were estimated from the buoyant density of the vesicles by assuming the buoyant density of 1.24 g/mL for the protein, obtained from density-gradient centrifugation of lipid-depleted p.m. (Hwang & Stoeckenius, 1977), and 1.06 g/mL for the lipid (Nagle & Wilkinson, 1978). The concentrated vesicle suspension (1 mL) was layered on top of a 10.5-mL, 10–45% sucrose density gradient and centrifuged at 200000*g* for 26–36 h at 5 °C. Fractions were collected and the sucrose concentration was measured with a Bausch and Lomb Abbe-3L refractometer.

Retinal was extracted with $CH_2Cl_2$ as previously reported, and the isomer concentration was determined by high-pressure liquid chromatography (Pettei et al., 1977). $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described (Oesterhelt & Stoeckenius, 1971).

*Preparation of Modified Purple Membrane.* Purple membrane was extensively cross-linked by suspending it for 8 h at room temperature in 2% glutaraldehyde, buffered with 50 mM cacodylate buffer at pH 6.9. The glutaraldehyde was then removed by washing 4 times with ice-cold water. DOC and Triton X-100 treatments of purple membrane have been described (Casadio et al., 1980; Hwang & Stoeckenius, 1977). Glutaraldehyde- and DOC-treated washed p.m. (6 mg) was added to a suspension of 60 mg of egg lecithin in 3 mL of 5% DOC, pH 7.8, to reconstitute cross-linked p.m. with lipid, and the preparation was dialyzed against 6.6 mM phosphate buffer, pH 6.0, for several days at 4 °C.

*Preparation of Lipid/Bacteriorhodopsin Vesicles.* For the preparation of vesicles a freeze–thaw sonication technique was used as described (Kasahara & Hinkle, 1977) with the following modifications. The lipids were dried from chloroform/methanol (4:1 v/v) as a thin layer in a 50-mL round-bottom flask and resuspended in 3–5 mL of 200 mM KCl buffered with 6.6 mM potassium phosphate at pH 6. The suspension was flushed with nitrogen and sonicated in a bath-type sonicator (Laboratory Supplies Co., Inc., Hicksville, NY, Model T 80-80-1) for ~2 h. Usually, to half of the lipid suspension was added the purple membrane (2 mg) to give a final volume of 3 mL. The other half was used as a reference sample for the spectroscopy after adding the appropriate amount of water. Both samples were then quickly frozen in a dry ice/ethanol bath, thawed at room temperature in ~5 min, and sonicated for 2–4 min above the phase-transition temperature of the lipid used. Absorption spectra indicated that no significant destruction of the chromophore had occurred.

Many different techniques for the incorporation of proteins into lipid vesicles have been developed. In addition to the freeze–thaw sonication technique (FTST), we have also tried the sonication cholate and deoxycholate techniques (Hwang & Stoeckenius, 1977) and the Triton dialysis technique (Cherry et al., 1978); however, the FTST was the simplest technique that gave consistent results. The vesicle suspensions, when analyzed on density gradients, usually gave broad single bands, but some pure lipid remained on top of the gradient. In general, the higher the lipid to protein ratio before sonication the lower was the density of the resulting lipid/bR vesicles. For instance, using FTST and DMPC at an initial weight ratio of 25:1 lipid/bR, we obtained a band with a buoyant density of 1.08–1.10 g/mL, while, with an initial ratio of 5:1 lipid/bR, the band had a buoyant density of 1.12–1.14 g/mL. This corresponds to molar lipid/bR ratios of 30:1 to 50:1 for the higher density and 80:1 to 150:1 for the lower density vesicles. In addition, the lipid concentration in the vesicles increases with the time of sonication as already shown (Hellingwerf et al., 1978). However, prolonged sonication leads to the destruction of the chromophore (Hellingwerf et al., 1978; Hwang & Stoeckenius, 1977). In this respect the FTST is a much better technique than simple sonication, because it requires much shorter sonication times to achieve the same degree of incorporation so that no significant bleaching is observed even at high lipid/bR ratios. We have used throughout this series of experiments the initial ratio of lipid to protein as a measure for the relative ratios in the vesicles. Only in representative experiments did we analyze the preparations on sucrose density gradients.

At high lipid concentrations the preparations scatter light strongly, and scattering changes also occur upon light adaptation of bR/lipid vesicles. Fortunately, the scattering changes do not significantly affect the position of the absorbance maximum. In all cases we find blue shifts of the absorbance maxima of up to 10 nm when we replace the native lipids. This is especially obvious above the phase transition of the substituting lipids. The extent of the blue shift generally increases with the amount of lipid incorporated and with increasing temperature. It is difficult to separate the influence of the lipid environment, of the lattice dissociation, and of the temperature on these blue shifts. The lattice-dissociation contribution is probably small as we also observed in Triton-solubilized p.m. (Casadio et al., 1980).

*Light Adaptation.* Completely dark-adapted samples in a thermostated holder were exposed to light from a slide projector equipped with a 250-W quartz iodine lamp, a heat filter, and a Corning glass long pass filter, 3-68. The light intensity at the holder was $3.0 \times 10^5$ erg $cm^{-2}$ $s^{-1}$, measured with a Model 68 Kettering radiometer (Laboratory Data Control Division, Milton Roy Co.). Light-adaptation times were from 3 to 6 min. In all cases we determined that longer exposure to light did not increase the red shift of the absorbance maximum. Immediately after illumination, several spectra were recorded in rapid succession to exclude the possibility that a rapid dark adaptation might affect our measurement of light adaptation. We also checked for an actinic effect of the measuring beam and found none. Monomeric bR, especially at elevated temperatures, is subject to photobleaching, so that a fully light-adapted sample may actually show a decrease of a few percent absorbance (Casadio et al., 1980). The photobleaching, however, does not affect the red shift, and, therefore, throughout the work, only the red shift is used as a measure of light adaptation. At elevated temperatures this photobleaching may be rapidly reversible, so that the second and third scan of the spectra after light adaptation may give slightly higher absorbances than the first. Again the red shift is unaffected. In the figures presented here we have used the first spectrum recorded.

## Results

(*1*) *Cross-Linked Purple Membrane.* Figure 1A shows the spectra of light-adapted and dark-adapted p.m. extensively cross-linked with glutaraldehyde and subsequently treated with

Table I: Light–Dark Adaptation in Triton-Treated p.m.

| prepn[a] | $\lambda_{max}$ (nm) DA | $\lambda_{max}$ (nm) LA | isomer compn[b] (13-cis:all-trans) DA | isomer compn[b] (13-cis:all-trans) LA | CD[c] | red shift (nm) | retinal isomerization[d] |
|---|---|---|---|---|---|---|---|
| (1) p.m. | 559 ± 1 | 569 ± 1 | (45 ± 1):(55 ± 1) | (18 ± 1):(82 ± 1) | + | 10 | 60 ± 2 |
| (2) p.m., Triton-treated | 549 ± 1 | 553 ± 1 | (58 ± 1):(42 ± 1) | (38 ± 1):(62 ± 1) | − | 4 | 34 ± 2 |
| (3) p.m., glutaraldehyde-treated | 558 ± 1 | 566 ± 1 | (57 ± 1):(43 ± 1) | (20 ± 1):(80 ± 1) | + | 8 | 64 ± 2 |
| (4) p.m., glutaraldehyde-treated in Triton | 551 ± 1 | 559 ± 1 | (63 ± 1):(37 ± 1) | (23 ± 1):(77 ± 1) | + | 8 | 63 ± 2 |
| (5) p.m., glutaraldehyde-treated, Triton-treated, and washed | 555 ± 1 | 559 ± 1 | n.d.[e] | n.d. | n.d. | 4 | n.d. |

[a] All experiments were carried out in 0.1 M sodium acetate buffer, pH 5, at 20 °C. [b] The yield of extracted retinal was 10–15% in all cases. In control experiments *all-trans*-retinal was added to the appropriate lipid suspension or buffer at the same temperature and recovered to 98% as the isomer was added. [c] (+) and (−) refer to the presence or absence of the 600-nm negative band in CD spectra. [d] Given as percent of 13-*cis*-retinal converted to *all-trans*-retinal upon light adaptation: $(13\text{-cis}_{DA}\text{-}13\text{-cis}_{LA}):13\text{-cis}_{DA} \times 100$. [e] n.d. = not determined.

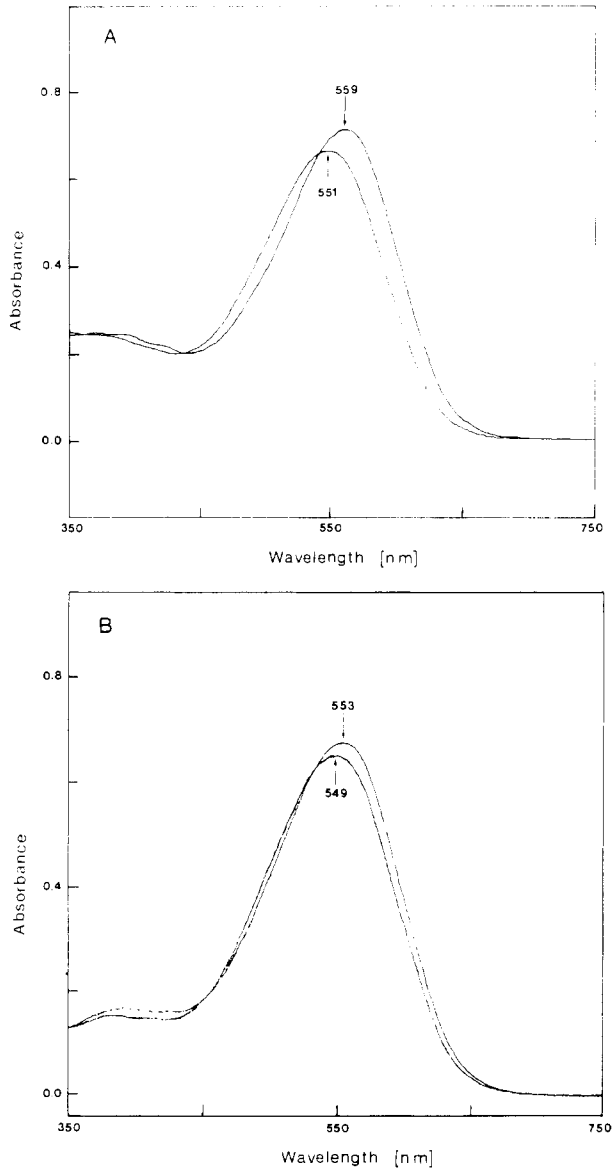


FIGURE 1: Spectra of light-adapted and dark-adapted p.m. in Triton X-100: (A) p.m. cross-linked with glutaraldehyde; (B) native p.m. For experimental conditions, see Table I and Materials and Methods.

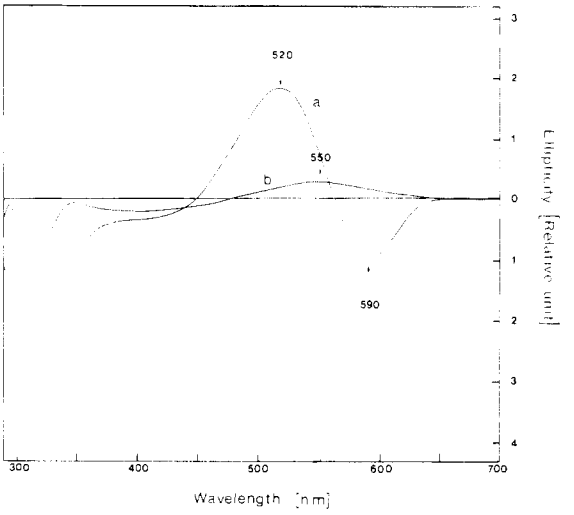


FIGURE 2: CD spectra of the preparations shown in Figure 1: (a) glutaraldehyde-treated p.m.; (b) native p.m.

Triton X-100 under conditions which completely solubilize native p.m. The extent of the red shift is slightly reduced from 10 nm in native p.m. to 8 nm in the cross-linked preparation; however, the same 20% reduction in the extent of the red shift is also present in the cross-linked membrane before Triton treatment. The only prominent effect of Triton is an 8 ± 1 nm blue shift of the absorbance maximum for both the dark-adapted and the light-adapted p.m. This 8-nm light adaptation compares to a 4-nm red shift in the solubilized p.m. (Figure 1B). The presence of a negative CD band at ~600 nm as well as X-ray diffraction (not shown) establishes that cross-linking protects the lattice structure against solubilization by Triton (Figure 2). These results are consistent with the conclusion that maintenance of the lattice structure is essential for a large light-adaptation effect (see Table I) and also confirm our earlier observations that Triton X-100, independent of its effect on the lattice, causes a blue shift of the absorbance maximum (Casadio et al., 1980). However, Triton may separate the p.m. lipids from bR more effectively when the lattice is dissociated than when it is kept intact by cross-linking, and thus we cannot conclusively exclude a lipid effect on light adaptation. We have therefore carried out a series of experiments with lipid-extracted cross-linked p.m.

It is known that exposure to DOC removes more than 80% of the lipid from p.m. but leaves the bR lattice intact. The treatment shifts the absorbance maximum to 555 nm in the dark- and 562 nm in the light-adapted state (Hwang & Stoeckenius, 1977). The blue shift is apparently a detergent effect on the chromophore, because, when DOC and the solubilized membrane lipids are washed out, $\lambda_{max}$ of the dark-adapted preparation returns to 558 nm. In the presence of DOC, light adaptation is reduced to a 7-nm red shift, and,

Table II: Light–Dark Adaptation in DOC-Treated p.m.

| prepn[a] | pH | $\lambda_{max}$ (nm) DA | $\lambda_{max}$ (nm) LA | isomer compn[b] (13-cis:all-trans) DA | isomer compn[b] (13-cis:all-trans) LA | CD[b] | red shift (nm) | retinal isomerization[b] |
|---|---|---|---|---|---|---|---|---|
| (1) p.m. | 7.0 | 559 ± 1 | 569 ± 1 | (45 ± 1):(55 ± 1) | (9 ± 1):(91 ± 1) | + | 10 | 80 ± 2 |
| (2) p.m., glutaraldehyde-treated | 7.0 | 558 ± 1 | 567 ± 1 | (60 ± 2):(40 ± 2) | (25 ± 2):(75 ± 2) | + | 9 | 58 ± 4 |
| (3) p.m., DOC-treated and washed | 6.9 | 558 ± 1 | 561 ± 1 | (43 ± 2):(57 ± 2) | (31 ± 2):(69 ± 2) | + | 3 | 28 ± 4 |
| (4) p.m., glutaraldehyde-treated in DOC (10%) | 7.8 | 556 ± 1 | 562 ± 1 | n.d. | n.d. | + | 6 | n.d. |
| (5) same as (4), DOC washed out | 6.1 | 559 ± 1 | 562 ± 1 | (38 ± 1):(62 ± 1) | (22 ± 3):(78 ± 3) | + | 3 | 42 ± 7 |
| (6) same as (5), in DOC (1%) | 7.0 | 557 ± 1 | 562 ± 1 | (48 ± 1):(52 ± 1) | (19 ± 1):(81 ± 1) | + | 5 | 60 ± 2 |
| (7) same as (5), in Triton (1%) | 5.0 | 553 ± 1 | 561 ± 1 | (57 ± 1):(43 ± 1) | (21 ± 1):(79 ± 1) | + | 8 | 63 ± 2 |

[a] (1), (2), (3), and (5) carried out in 50 mM cacodylate buffer at the pH indicated, (4) and (6) in deoxycholic acid with the pH adjusted with NaOH, and (7) in 0.1 M sodium acetate. The temperature was 20 °C. [b] See Table I.

after washing out the DOC, to a 3-nm red shift. These effects of DOC do not change significantly if the membrane is first cross-linked with glutaraldehyde (Table II). Now, however, we can add Triton to the lipid-depleted preparation without dissociating the lattice. The result is a blue-shifted $\lambda_{max}$ as expected from the detergent effect on the chromophore, but the red shift upon light adaptation is now 8 nm, i.e., the same value observed in unextracted cross-linked p.m. If instead of Triton we add back DOC, we get a smaller blue shift of $\lambda_{max}$ and a 5-nm red shift upon light adaptation. The extent of this light adaptation is, however, nearly twice the value observed in the lipid-extracted membrane in the absence of DOC. The presence of the negative CD band shows that in all of these preparations the bR lattice has been preserved. We conclude that the presence of the lattice alone is not sufficient to maintain the full extent of light adaptation; a lipid environment is required. However, the lipid requirement is rather unspecific; Triton is fully as effective as the native lipids, and even DOC restores a substantial part of the light adaptation. Line 5 of Table I may actually also demonstrate this, because phosphorus analysis of this preparation showed that Triton removed the native membrane lipids as effectively as DOC (data not shown). This still leaves the objection that glutaraldehyde treatment may inhibit an effect of Triton on the protein, which in native p.m. causes the reduction of light adaptation.

*(2) Lipid Vesicles.* The lipid environment should be kept as constant as possible to further explore the effect of lattice structure on light adaptation. In intact p.m. the bR lattice dissociates at temperatures > 80 °C (Jackson & Sturtevant, 1978a,b). Unfortunately, the dissociation is far from complete (S.-B. Hwang and W. Stoeckenius, unpublished experiments); the dark adaptation and also reversible photobleaching processes become very fast at this temperature so that they cannot be easily measured and separated. However, if bR is incorporated into lipid vesicles with a high lipid to protein ratio, it exists in the form of monomers above the phase transition and reaggregates when the temperature is decreased to below the phase-transition temperature of the lipid ($T_c$) (Cherry et al., 1978; S.-B. Hwang and W. Stoeckenius, unpublished experiments). To eliminate possible differences caused by the state of the lipids, we can again use cross-linking to prevent dissociation of the lattice above the phase transition and compare, for instance, light adaptation in egg lecithin/bR vesicles prepared from native and glutaraldehyde cross-linked p.m. at 10 °C. The bR-containing material was reisolated on a sucrose density gradient, where the cross-linked preparation gave a narrow band with a buoyant density of 1.14 g/mL. When light adapted, it showed a red shift of 10 nm (from 551 to 561 nm), whereas the material not cross-linked showed only a 5-nm red shift (from 549 to 554 nm). These results are therefore consistent with our earlier conclusion that the lattice structure is necessary for full light adaptation. However, the density-gradient centrifugation showed that the cross-linked material had taken up little more than twice the amount of lipid present in the native membrane and had a molar lecithin/bR ratio of 32:1, while the control preparation had taken up much more. We would also not expect much more than the native amount of lipid to be incorporated into the cross-linked lattice. If this part of the lipid were immobilized, the bR could still be in a rather rigid lipid environment, i.e., the lattice effect on light adaptation might not result from protein–protein interaction but could rather be mediated through an effect of the lattice structure on the lipid state which in turn would influence the light adaptation of bR. Even if we ignore this perhaps somewhat contrived objection, we are still left with another problem. Lipid vesicles formed from native p.m. with lipid/bR ratios in the same range as the cross-linked preparation even at temperatures above the lipid-phase transition do not show the large reduction in light adaptation characteristic of the monomer (Table III). This is apparently due to incomplete dissociation of the lattice, which to be complete requires higher lipid/bR ratios. We have therefore carried out additional experiments with lipid vesicles, designed to eliminate these objections. We have mainly used DMPC for the preparation of our lipid/bR vesicles, because it is easily available in high purity and has a sharp phase transition at $T_c$ = 23.5 °C (Chapman et al., 1967) so that extreme temperatures can be avoided in work both above and below the phase transition. Parallel experiments were carried out with DPPC ($T_c$ = 41.0 °C) (Chapman et al., 1967) and gave es-

Table III: Effect of Lipid to Protein Molar Ratio on Light–Dark Adaptation

| DMPC to bR molar ratio[a] | $T$ (°C) | $\lambda_{max}$ (nm) DA | $\lambda_{max}$ (nm) LA | CD[b] | red shift (nm) |
|---|---|---|---|---|---|
| 30:1 to 50:1 | 10 | 555 ± 1 | 564 ± 1 | + | 9 |
| | 35 | 553 ± 1 | 562 ± 1 | +[c] | 9 |
| 80:1 to 150:1 | 10 | 552 ± 1 | 561 ± 1 | + | 9 |
| | 35 | 549 ± 1 | 553 ± 1 | − | 5 |

[a] Ratios calculated for the upper and lower edges of the band in the 10–45% sucrose density gradient. [b] See Table I. [c] Reduced in extent.

Table IV: Light-Dark Adaptation in Lipid/bR Vesicles

| prepn[a] | lipid/bR[b] | $T$ (°C) | $\lambda_{max}$ (nm) DA | $\lambda_{max}$ (nm) LA | isomer compn[c] (13-cis:all-trans) DA | isomer compn[c] (13-cis:all-trans) LA | CD[c] | red shift (nm) | retinal isomerization[c] |
|---|---|---|---|---|---|---|---|---|---|
| (1) p.m. | 1:3 | 10 | 558 ± 1 | 568 ± 1 | (48 ± 1):(52 ± 1) | (21 ± 1):(79 ± 1) | + | 10 | 56 ± 2 |
| | | 35 | 556 ± 1 | 566 ± 1 | (46 ± 4):(54 ± 4) | (21 ± 1):(79 ± 1) | + | 10 | 54 ± 2 |
| (2) p.m. plus DMPC | 25:1 | 10 | 551 ± 1 | 560 ± 1 | (48 ± 1):(52 ± 1) | (20 ± 1):(80 ± 1) | + | 9 | 58 ± 2 |
| | | 35 | 549 ± 1 | 553 ± 1 | (46 ± 1):(54 ± 1) | (37 ± 1):(63 ± 1) | − | 4 | 19 ± 2 |
| (3) p.m. plus DMPC | 400:1 | 10 | 550 ± 1 | 555 ± 1 | (48 ± 2):(52 ± 2) | (34 ± 2):(66 ± 2) | n.d. | 5 | 29 ± 2 |
| | | 35 | 547 ± 1 | 551 ± 1 | n.d. | n.d. | n.d. | 4 | n.d. |
| (4) p.m. plus azolecithin | 25:1 | 10 | 548 ± 1 | 553 ± 1 | (48 ± 1):(52 ± 1) | (34 ± 1):(66 ± 1) | − | 5 | 29 ± 2 |

[a] All experiments were carried out in 200 mM KCl (and 6.6 mM phosphate buffer, pH 5.8, with the FTST. [b] Weight ratio; lipid refers to the lipid added. Except for line 1 the lipids present in p.m. were neglected. [c] See Table I.

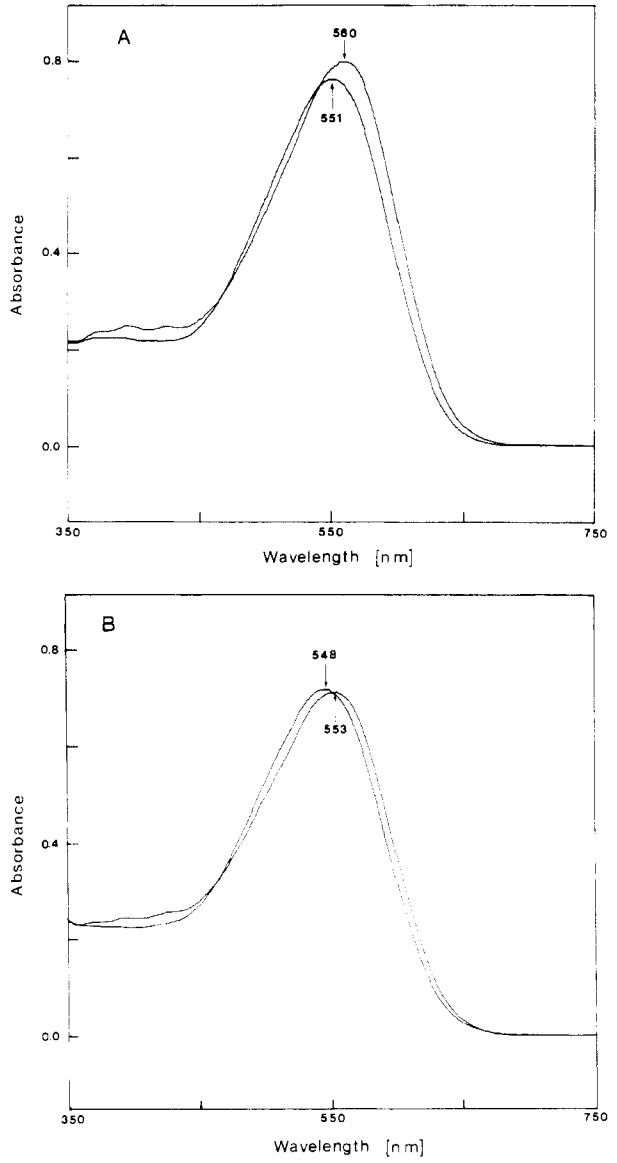


FIGURE 3: Spectra of light-adapted DMPC/bR vesicles prepared with a weight ratio of 25:1: (A) at 10 °C; (B) at 35 °C.

sentially the same results, taking into account the difference in $T_c$ for the two lipids.

Table IV shows that, at a DMPC/bR weight ratio of 25:1, light adaptation results in a 9-nm red shift at 10 °C and a 4-nm red shift at 35 °C (Figure 3). The CD spectra of the same preparation show that, as expected, the protein is aggregated at the lower temperature and monomerically dispersed at the higher temperature (Figure 4). The experiment with azolecithin (see Table IV) rules out that this is simply

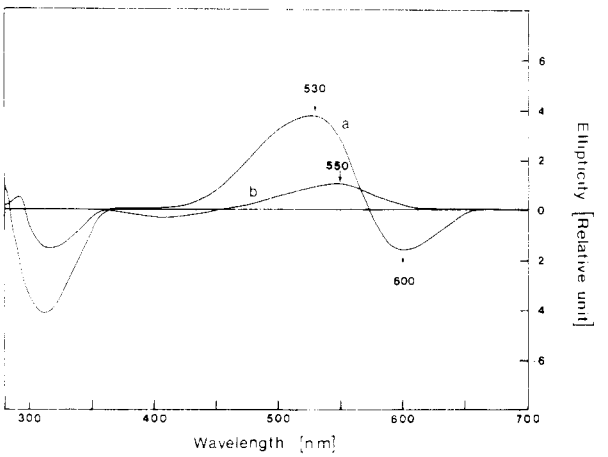


FIGURE 4: CD spectra of the same vesicles as in Figure 4: (a) at 10 °C; (b) at 35 °C.

Table V: Temperature Effect on Light-Dark Adaptation of DPPC/bR Vesicles[a]

| $T$ (°C) | $\lambda_{max}$ (nm) DA | $\lambda_{max}$ (nm) LA | red shift (nm) |
|---|---|---|---|
| 10 | 550 ± 1 | 559 ± 1 | 9 |
| 15 | 549 ± 1 | 558 ± 1 | 9 |
| 35 | 547 ± 1 | 555 ± 1 | 8 |
| 45 | 548 ± 1 | 552 ± 1 | 4 |
| 55 | 547 ± 1 | 549 ± 1[b] | 2 |

[a] The DPPC/bR vesicles were prepared according to Cherry et al. (1978). The lipid to protein molar ratio was in the range of (110–160):1. [b] At this temperature, the dark adaptation becomes so fast that the first spectrum scanned is no longer fully light adapted.

an effect of the temperature, because in this preparation, which is above its phase transition at 10 °C, we observe the 4-nm red shift characteristic of the monomer. Conversely, DPPC vesicles at 35 °C, i.e., below their phase transition, show an 8-nm red shift (Table V) and a negative CD band. It would, however, be still more convincing if we could obtain bR monomers and aggregates in exactly the same lipid environment. Experiment 3 of Table IV is designed to achieve this condition. Obviously, if a sufficiently high lipid/bR ratio is used, most bR molecules should be trapped as monomers in a solid lipid phase when the preparation is rapidly cooled through the phase transition. Moreover, if only one bR molecule is present in a lipid vesicle, aggregation is impossible even at slow cooling rates. The decrease in visible absorbance and the large increase in scattering make working with high lipid/bR ratios very difficult. The DMPC/bR weight ratio of 400:1 was the highest we could use and still measure light adaptation reliably. Ideally, with an average vesicle diameter of ~600 Å (determined by electron microscopy), this should yield a preparation

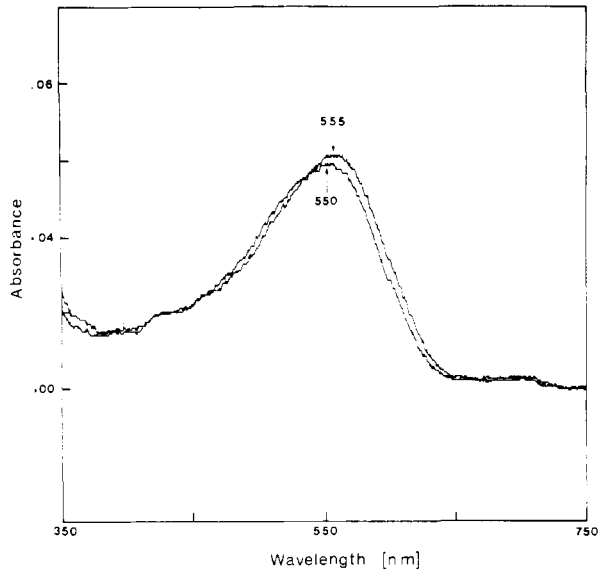


FIGURE 5: Spectra of light-adapted and dark-adapted DMPC/bR vesicles at 10 °C. The initial weight ratio of lipid to protein was 400:1. Because of the necessarily very low concentration of bR in the sample, the quality of the spectra is not as good as in the other preparations.

containing on the average less than two molecules of bR per vesicle. Presumably the preparation was not homogeneous, and the high degree of light scattering did not allow us to verify the aggregation state of bR with the CD spectrophotometer available to us. Nevertheless, we see that in this preparation below the phase transition at 10 °C we obtain a red shift of only 5 nm (Figure 5), which within our error limits is the same as that obtained with the same preparation (or the 25:1 preparation) at 35 °C. We conclude that we have succeeded in trapping most of the bR as monomers below the lipid-phase transition and that the bR monomers in a solid lipid environment show the same reduced light adaptation as monomers in a liquid-crystalline lipid phase.

*Isomer Configuration of Retinal.* In our earlier investigation of light adaptation in Triton-solubilized preparations, we have shown that the decreased extent of light adaptation in bR monomers is also characterized by decreased conversion of cbR to tbR (Casadio et al., 1980). The isomer composition for most of the preparations described here is included in the tables. The decrease in the red shift in all cases is paralleled by a decreased isomerization of 13-*cis*- to *all-trans*-retinal. We consider the extraction data less reliable than the red-shift measurements. The standard deviations given are for several determinations of the same extract. Differences between extractions are considerably larger and may amount to as much as ±10%. In any case, conversion of 13-*cis*- to *all-trans*-retinal in the photocycle above the lipid-phase transition, or below the phase transition if bR is present in the monomeric state, is reduced at least as much as it is in Triton-solubilized monomers. However, when bR is aggregated, the conversion of 13-cis to all-trans is as high as in intact p.m. We assume that the cause for the decreased conversion in lipid vesicles is also the same, i.e., a back-reaction of an intermediate in the tbR photocycle to cbR or one of its photocycle intermediates, because we have preliminary evidence that the bR monomers in lipid vesicles also show a wavelength dependence of light adaptation similar to what we have found in Triton-solubilized bR (Casadio et al., 1980).

## Discussion

We have demonstrated earlier that the bR monomer, obtained by solubilization of p.m. in Triton X-100, shows a reduced extent of light adaptation measured as the extent of the red shift of the absorbance maximum, the increase in absorbance, or the isomerization of 13-*cis*- to *all-trans*-retinal (Casadio et al., 1980). The results presented here show that the same holds true for the bR monomer in phospholipid bilayers, with a molar lipid/bR ratio ≥ 80:1 above their phase-transition temperature. If bR is reaggregated by lowering the temperature of such a preparation below the lipid-phase transition, the extent of light adaptation is restored to the same or almost the same value found in intact p.m. Protein–protein interactions and not the state of the lipid bilayer are apparently responsible for the high extent of light adaptation in the purple membrane lattice, because, if the reassociation is prevented by using very high lipid/bR ratios, we observe the low extent of light adaptation characteristic for the monomer even below the lipid phase transition temperature. The lipid environment is not completely without influence on the extent of light adaptation, because in p.m., lipid-extracted with DOC, the lattice is retained but the extent of light adaptation is reduced to about the same level found in the monomer. However, the lipid requirement is not very specific; even the presence of DOC increases light adaptation, and Triton X-100 or egg lecithin fully restores it. We may note here that water is also required, because drying the membrane in air also reduces and finally abolishes light adaptation (Korenstein & Hess, 1977) while the order of the bR lattice is maintained and the unit cell dimension is reduced by less than 2% (Blaurock & Stoeckenius, 1971).

In Triton-solubilized monomers, light adaptation results from the photo steady state of cbR and tbR reaction-cycle intermediates, which develops upon illumination (Casadio et al., 1980). The same should hold true for the monomers in lipid bilayers, because they show the same wavelength dependence of the extent of light adaptation (our unpublished experiments). In the aggregated state of bR, i.e., in the intact p.m. or in lipid vesicles below the phase transition, protein–protein interactions apparently shift the photo steady state toward tbR. A decrease in the decay rate of photocycle intermediate M in monomeric bR is consistent with this explanation (S.-B. Hwang and W. Stoeckenius, unpublished experiments). The shift toward tbR should increase the efficiency of light-energy conversion in the cell, because only the tbR photoreaction cycle pumps protons (Ohno et al., 1977; Lozier et al., 1978). In addition, the aggregated bR is much less susceptible to permanent bleaching by high light intensities. The thermal equilibrium, which determines the ratio of cbR to tbR in the dark-adapted state, is less influenced by protein–protein interactions. We found a small increase in the rate of dark adaptation (Casadio et al., 1980), and the cbR/tbR ratio is slightly higher than in p.m. or lipid vesicles below the phase transition. We have not observed the large increase in the dark-adaptation rate reported for bR monomers in bleached and partially reconstituted p.m. (Ebrey et al., 1977).

A rigorous analysis of the aggregation state of bR in lipid vesicles is presently not possible. Electron micrographs of a typical DMPC/bR vesicle preparation, with a molar lipid/bR ratio of 80–150, show, when frozen from below the phase transition, mainly small aggregates of particles and no well-ordered extended lattice; the tendency to form parallel equidistant rows of particles, which Cherry et al. (1978) observed in their preparations of larger vesicles, is also obvious. The main difference to preparations frozen from above the phase transition is a less uniform distribution of particles over the fracture face (Figure 6). The particles do not correspond to

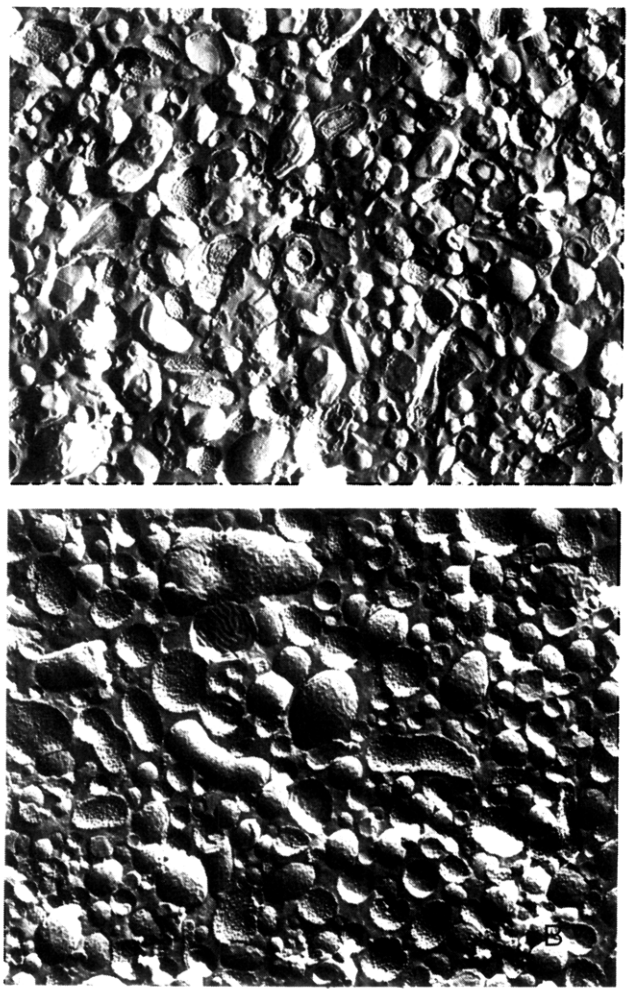

FIGURE 6: Freeze–fracture electron micrographs of DMPC/bR vesicles (25:1 w/w). The magnification is 70 000×. (A) Vesicles frozen from below the phase-transition temperature of the lipid. The bR molecules are aggregated and unevenly distributed over the fracture face. A tendency to form rows of particles is obvious. Note the faceted appearance of the vesicles, characteristic for the gel-like state of the lipid. (b) Vesicles frozen from above the phase-transition temperature of the lipid. The particles are more uniformly distributed over the fracture face but are less uniform in size.

single molecules, which cannot be resolved by the replication technique (Fisher & Stoeckenius, 1977). In preparations quenched from above the phase-transition temperature, the particles presumably arise from collisions of bR molecules during quenching; diffusion in liquid-crystalline lipid bilayers is fast enough to allow such collisions to occur even at the fastest cooling rates achieved with conventional techniques (Cherry et al., 1978). However, the existence of small irregular aggregates not numerous or ordered enough to give detectable exciton interaction cannot be excluded. Below the phase-transition temperature bR aggregates must already exist and could presumably only remain unchanged or become larger during quenching. Since the 100-Å particles seen in electron micrographs from preparations below the phase transition do not appear in well-ordered aggregates, they themselves presumably give rise to the observed exciton interaction. Their size indicates that they may be nonamers or dodecamers (Fisher & Stoeckenius, 1977).

The question arises as to why bR is crystalline in its native lipid environment. These native lipids are presumably above their phase transition under physiologic conditions, if such a transition exists at all, and their viscosity should be lower than that of DPPC (Jackson & Sturtevant, 1978a,b; Lindsey et al., 1979). Obviously the protein is not immobilized; the p.m. lattice is disordered by the removal of retinal, and reconstitution of the chromophore with retinal restores the lattice structure (Hiraki et al., 1978). This could be due to the relatively high protein concentration; however, in the brown membrane, which is a precursor of p.m., the retinal-free apoprotein of bR is present, dispersed and in low concentrations. If retinal is added to this membrane, bR forms and aggregates spontaneously (Hiraki et al., 1978; Hwang et al., 1979; Sumper et al., 1976; Danon et al., 1977). The native lipid, therefore, must induce lattice formation by an interaction different from that observed in the lipids used in this study, which may simply be a concentration effect resulting from a phase separation below the phase-transition temperature.

The amount of cbR we find in aggregated and fully light-adapted bR and p.m. is higher than that usually reported in the literature [e.g., Oesterhelt et al. (1973) and Sperling et al. (1977)]. This may in part be due to the relatively acidic pH of our preparations because the ratio of cbR to tbR increases with decreasing pH (Mowery et al., 1979). However, even in intact p.m. at pH 7.0 we find 9% cbR. This is independent of variations in our extraction technique, and the discrepancy with literature values remains unexplained. We believe that the data are not an artifact and that a significant back-reaction from the tbR the cbR cycle may exist even in intact p.m. at neutral pH. However, the amount of residual cbR in aggregated, light-adapted preparations does not affect our conclusions, and the light/dark-adaptation mechanism may in the meanwhile serve as a useful probe for the aggregation state of bR.

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# In Vitro Inhibition of Vitamin K Dependent Carboxylation by Tetrachloropyridinol and the Imidazopyridines†

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ABSTRACT: The compounds 2,3,5,6-tetrachloro-4-pyridinol (TCP) and the structurally related imidazopyridines (IP) cause hemorrhage and lower the plasma prothrombin level in animals. In vitro, TCP and the IP are more potent inhibitors of both the vitamin K dependent carboxylase which catalyzes the posttranslational γ-carboxylation of specific glutamyl residues in proteins and the related vitamin K epoxidase activity than they are either of vitamin K epoxide reductase or of NAD(P)H-K oxidoreductase. TCP and IP, as is the case with the coumarin and indandione anticoagulants, are competitive inhibitors of NAD(P)H-K oxidoreductase with respect to NADH. The epoxide reductase from coumarin-resistant rats is quite resistant to inhibition not only by warfarin but also by the IP, and to a lesser extent by TCP. When interpreted in light of published in vivo experiments, the data suggest that the principal site of anticoagulant action of the IP, but not TCP, is the epoxide reductase. The anticoagulant effect of TCP may be inhibition of the carboxylase itself. TCP is a significantly more potent inhibitor of the carboxylase and epoxidase than the IP; it inhibits both the enzymatic activities to the same degree with 50% inhibition observed at about $10^{-5}$ M. Inhibition of the carboxylase by TCP is not competitive with respect to the pentapeptide substrate phenylalanylleucylglutamylglutamylleucine nor with respect to the following components of the in vitro carboxylase assay: imidazole, pyridoxal 5′-phosphate, dithiothreitol, KCl, sodium bicarbonate, oxygen, and vitamin K. The order of addition of components of the assay relative to the addition of inhibitor did not affect the degree of inhibition. Inhibition is readily reversed in experiments designed to dissociate an enzyme–inhibitor complex. Analysis of double-inhibitor experiments suggests that TCP and IP have the same binding site on the carboxylase.

The posttranslational γ-carboxylation of specific glutamyl residues in proteins (Stenflo et al., 1974; Nelsestuen et al., 1974) is catalyzed by a vitamin K dependent carboxylase (Esmon et al., 1975) which is located in the endoplasmic reticulum (Helgelund, 1977). The basic requirements of the carboxylase, which has not yet been purified extensively, include oxygen, $CO_2$ or bicarbonate, and the hydroquinone form of the vitamin (Esmon et al., 1975; Friedman & Shia, 1976; Jones et al., 1976; Girardot et al., 1976). In addition to the carboxylase, several other enzymes are required for the normal in vivo function of vitamin K. In particular, NAD(P)H-K oxidoreductase ("DT-diaphorase", EC 1.6.99.2) or a very similar enzyme is required for the reduction of vitamin K quinone to the active hydroquinone (Wallin et al., 1978). In vivo and during in vitro incubations with microsomes prepared from a variety of tissues, vitamin K is converted enzymatically to a 2,3-epoxide (Matschiner et al., 1970; Willingham & Matschiner, 1974). Although epoxidation appears to be associated with the vitamin K dependent carboxylation event (Willingham & Matschiner, 1974; Willingham et al., 1976; Friedman & Smith, 1977, 1979), coupling of the two reactions has not been demonstrated. The epoxide itself is inactive in supporting carboxylation, yet another enzyme, vitamin K epoxide reductase, also located in the endoplasmic membrane, will reduce the epoxide to the quinone form of the vitamin (Matschiner et al., 1974; Zimmerman & Matschiner, 1974; Willingham & Matschiner, 1974; Whitlon et al., 1978).

The anticoagulants, the coumarins and indandiones, are potent inhibitors of both the DT-diaphorase (Ernster et al., 1962) and the epoxide reductase activities (Bell & Caldwell,

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