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Insertion of bacteriorhodopsin into polymerized diacetylenic phosphatidylcholine bilayers

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We have developed a method to incorporate the membrane protein bacteriorhodopsin into polymerized bilayers composed of a diacetylenic phosphatidylcholine, 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) and a non-polymerizable phospholipid, dinonanoylphosphatidylcholine (DNPC). The extent of $DC_{8,9}PC$ polymerization in the bilayer was significantly improved when 2:1 mole ratio DNPC-$DC_{8,9}PC$ was used. Octyl glucopyranoside-solubilized bacteriorhodopsin was inserted into the polymerized DNPC-$DC_{8,9}PC$ bilayers by overnight incubation at 4°C followed by dialysis to remove the detergent. The protein was inserted into the membranes after photo-polymerization to avoid inactivation of the protein due to the UV irradiation. The insertion of bacteriorhodopsin into the polymerized DNPC-$DC_{8,9}PC$ membranes was confirmed by density gradient centrifugation, UV/visible spectroscopy, and freeze fracture electron microscopy. The polymerized DNPC-$DC_{8,9}PC$ membranes containing bacteriorhodopsin were about 10% protein by weight. These results suggest that mixed lipid systems such as the DNPC-$DC_{8,9}PC$ can be used to improve both the extent of polymerization and the efficiency of membrane protein incorporation in the polymerized bilayer.

Introduction

Reconstitution of integral membrane proteins in phospholipid bilayers is one of the best methods for studying the structure and function of proteins in biological membranes. These studies have been done almost exclusively with naturally occurring phospholipids. However, lipids are now available that contain cross-linking groups. For example, lipids have been synthesized which contain diacetylenic [1,2,3], methacryloyl [4,5], and dienic [6,7] groups. In principle, these lipids can be polymerized by UV irradiation to form chains of covalently linked lipids in the bilayer. Polymerizable lipids may be useful for the fabrication of protein containing membranes with enhanced physical and chemical stability. We are developing procedures to reconstitute membrane proteins into rugged phospholipid bilayers formed from such novel lipids. Polymerized membranes containing proteins have attracted considerable interest for potential applications such as solar energy conversion, bio-compatible surfaces, drug encapsulation, and biosensors [8].

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Abbreviations: $DC_{8,9}PC$, 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine. Our convention is to abbreviate the name of the diacetylenic phosphatidylcholines as $DC_{m,n}PC$. The position of the diacetylenic group in the alkyl chain is indicated by *m* and *n* where *m* equals the number of methylene segments between the carbonyl carbon and the first diacetylene carbon and *n* equals the number of methylene segments between the last diacetylene carbon and the terminal methyl group; a.u., absorbance unit; Bistris, bis(2-hydroxyethyl) imino-tris(hydroxymethyl)methane; bR, bacteriorhodopsin; DENPC, 1,2-bis(octadeca-2,4-dienoyl)phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DNPC, dinonanoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearylphosphatidylcholine; eV, electron volts; MW, molecular weight; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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The diacetylenic phosphatidylcholine 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) was used in this study. The diacetylenic groups can form intermolecular cross-links to produce a highly conjugated polymer within the hydrocarbon region of the bilayer. Polymerizing $DC_{8,9}PC$ into long extended polymers should make the bilayer more rugged, i.e., more stable to mechanical and chemical perturbations. Bacteriorhodopsin (bR), the light-driven proton pump from the halophilic bacteria *Halobacterium halobium* [9] was used as a model membrane protein for this study. Bacteriorhodopsin is a 26000 MW integral membrane protein that is composed of seven trans-membrane α-helices.

There have been several previous reports of membrane protein reconstitution with polymerizable lipids [10,11,12]. In most experiments, the protein was incorporated into polymerizable lipid membranes followed by UV irradiation to polymerize the bilayer. Purple membrane fragments have been incorporated into polymerized membranes by co-sonication with diacetylenic lipid [12], and polymerizable sulfolipids [11]. In both experiments the UV irradiation used to initiate the polymerization of the bilayer was found to at least partially inactivate the protein. O'Brien and co-workers [13,14] avoided this problem by inserting rhodopsin directly into polymerized bilayers composed of a 1 : 1 mole ratio of 1,2-bis(octadeca-2,4-dienoyl)phosphatidyl choline (DENPC) and dioleoylphosphatidylcholine (DOPC).

The principal problem associated with reconstituting membrane proteins into polymerized membranes is how to obtain extensive membrane polymerization without destroying the protein function. This problem is particularly acute for diacetylenic lipids like $DC_{8,9}PC$ because the UV irradiation used to polymerize the membrane appears to destroy protein function [12]. Moreover, the amount of polymerization in the pure $DC_{8,9}PC$ bilayers is probably quite low with a relatively small amount of long chain length polymer [15]. The average chain length of the diacetylenic polymer in the membrane will have a very important influence on the properties of the polymerized bilayer. Incorporating proteins into a polymerized bilayer consisting of only dimers and trimers probably will not significantly improve the stability of the lipid/protein membrane. The polymerized bilayer should have, in order to improve the stability, not only a relatively low amount of non-polymerized monomer, but also a large amount of very long chain length polymer.

We have developed a membrane protein reconstitution procedure using $DC_{8,9}PC$ and a non-polymerizable lipid DNPC and produces *both* extensive polymerization of the membranes and efficient protein incorporation. The key feature of this procedure is based on the recent finding of Singh and Graber [16] that the addition of short acyl chain phosphatidylcholine to diacetylenic phosphatidylcholine significantly enhances the efficiency of polymerization. In this report, we demonstrate the following: (i) addition of stoichiometric amounts of DNPC significantly improves the polymerization efficiency of $DC_{8,9}PC$, and (ii) detergent solubilized bR is efficiently inserted into polymerized DNPC-$DC_{8,9}PC$ bilayers.

Experimental procedures

Materials

The dinonanoylphosphatidylcholine, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). The *n*-octyl β-D-glucopyranoside was purchased from Calbiochem (San Diego, CA). The BCA protein assay reagent was from Pierce Chemical Co. (Rockford, IL). The rest of the reagents were of the highest analytical grade. *Halobacterium halobium* strain ET1001 was a generous gift from Dr. Walther Stoeckenius of the University of California and was grown in a well-illuminated fermentor. Purple membrane was isolated and purified following the procedure of Brecher and Cassim [17]. Isolated purple membrane was stored at an approximate concentration of 1 mg/ml in 5 mM sodium azide at 4° C. The polymerizable diacetylenic lipid 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine was synthesized in our laboratory using the previously published procedures [18]. The purity of the lipid was checked by thin-layer chromatography. The monomeric lipid had a single spot on silica gel 60 F-254 (EM Science, Cherry Hill, NJ) using a chloroform/methanol/water (65 : 25 : 4, v/v/v) solvent system.

UV initiated polymerization of the membrane

The 2 : 1 mole ratio DNPC-$DC_{8,9}PC$, DMPC-$DC_{8,9}PC$ and DPPC-$DC_{8,9}PC$ and the $DC_{8,9}PC$ membranes were polymerized in an identical manner. A total of 15–18 mg of lipids were weighed out and dissolved in HPLC grade chloroform. The chloroform was removed under reduced pressure at 55° C using a rotary evaporator. The final trace amounts of chloroform were removed by overnight vacuum desiccation. Enough KCl buffer (120 mM KCl, 30 mM Bistris, 5 mM NaN_3, pH 6.5) was added to resuspend the lipids at a concentration of 12 mg/ml total lipid. The lipids were then bath sonicated at 50 to 55° C at full power for 20 to 60 min. The DNPC-$DC_{8,9}PC$ samples were sonicated until the lipid suspension was optically clear, which required about 20 min. The other samples required approx. 60 min of sonication. Thin-layer chromatography indicated that no appreciable lipid breakdown products were produced. These samples were slightly more turbid than the DNPC-$DC_{8,9}PC$ samples. Before UV irradiation the

samples were cooled to below 4° C by placing them in an ice bath. Cooling caused the $DC_{8,9}PC$ sample to become viscous and the DNPC-$DC_{8,9}PC$ samples to become turbid. The turbidity of the DNPC-$DC_{8,9}PC$ samples at 4° C is consistent with the size of the lipid vesicles we observed by electron microscopy.

The lipid membranes were polymerized by UV-irradiation in a Rayonet Photochemical reactor which had sixteen 75 watt Hg lamps (Southern New England Ultraviolet Co., Hamden, CT). The samples were held in a quartz sample holder which had a circulating cold water cooling jacket. The UV-initiated polymerization was carried out at 4 ± 2° C. The temperature of the sample during the UV initiated polymerization was measured using a small thermistor placed in the sample (Omega Engineering, Inc., Stmford, CT). Following 1 min of UV exposure the DNPC-$DC_{8,9}PC$ samples were dark blue, the DMPC-$DC_{8,9}PC$ samples were faint blue, the DPPC-$DC_{8,9}PC$ samples were faint purple, and the $DC_{8,9}PC$ samples were faint light red. The membranes used in the protein incorporation experiments were polymerized for a total of 4 min. If allowed to reach room temperature, the color of all the polymerized membrane samples irreversibly changed to orange. The UV/visible spectra of the polymerized membranes were recorded with a Perkin-Elmer Lambda 4C spectrophotometer equipped with a thermoregulated sample and reference cuvette holders. Unless otherwise indicated, the spectra of the samples were recorded at room temperature with a bandwidth of 1 nm.

Estimation of the relative extent of polymerization

The relative extent of polymerization after UV irradiation was estimated by the UV/visible absorbance spectra of the polymerized membranes dissolved in chloroform/methnol/water (65 : 25 : 4, v/v/v). Dissolving the membranes in this solvent eliminated absorption artifacts due to light scattering of the membranes. The polymer samples would not completely dissolve in pure chloroform. The UV/visible spectra were recorded with the Perkin Elmer spectrophotometer using 1 cm pathlength quartz cuvettes with teflon stoppers (NGS Precision Cell, Framingdale, NY). The polymer solution in chloroform/methanol/water was not turbid. The polymer from polymerized DNPC-$DC_{8,9}PC$ membranes dissolved in this solvent contained polymer component with an absorbance peak at approx. 535 nm. This polymer component was removed by filtering the solution through a 0.45 μm pore size teflon filter (Millipore, Bedford, MA).

Insertion of bacteriorhodopsin into the polymerized membranes

Solubilized bR was prepared by incubating purple membrane in 60 mM octyl glucopyranoside, 120 mM KCl, 30 mM Bistris, 5 mM NaN_3 (pH 6.5) for 4 h in the dark at room temperature. The insertion of solubilized bR into the polymerized diacetylenic bilayers was accomplished by overnight incubation of 0.2 ml of polymerized membranes (12 mg/ml total lipid) with 0.2 ml of octyl glucopyranoside solubilized bR (4.8 mg/ml protein) at 4° C. The protein was inserted into the phosphatidylcholine bilayer after polymerization to avoid the inactivation of the protein by the UV irradiation. Experiments with purple membranes showed that after 5 min of UV irradiation the absorbance peak at 570 nm due to bR disappeared. The inactivation of the protein due to UV irradiation was not dependent on chromophore, all-*trans*-retinal, remaining associated with the protein. Apo-membrane, which is purple membrane with the retinal removed, did not rebind all-*trans*-retinal after UV irradiation. The initial lipid to protein weight ratio was 2.5 : 1 and the octyl glucopyranoside concentration was 30 mM in the incubation buffer. The bR concentration was determined by the absorbance at 570 nm using an extinction coefficient of $6.4 \cdot 10^4$ $1 \cdot mol^{-1} \cdot cm^{-1}$ [19]. Control polymerized membranes were prepared in an identical manner with 60 mM octyl glucopyranoside KCl buffer which contained no protein. The samples were then dialyzed with several changes against 500–700-fold excess of KCl buffer containing approx. 0.5 g of Bio-Beads SM-2 (Bio-Rad, Richmond CA) at 4° C. The overnight incubation and the dialysis were done in the dark. The polymerized membranes were then purified by continuous glycerol density gradient centrifugation. The samples were layered on a 10 to 60% glycerol gradient and centrifuged overnight at 34000 rpm at 4° C in a swinging bucket rotor. The thirteen gradient fractions were collected using a peristaltic pump and a fraction collector. The relative amount of diacetylenic polymer in each fraction was measured by the sample absorbance at 530 nm after allowing the sample to reach room temperature.

Assay for protein content

Incorporation of bR into the polymerized membranes was demonstrated by SDS-PAGE and UV/visible spectroscopy. The SDS-PAGE was done using the discontinuous method of Laemmli [20] using a slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). The protein bands were visualized with Coomassie brilliant blue dye R-250 UV/visible spectroscopy. The SDS-PAGE was done using the discontinuous method of Laemmli [20] using a slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). The protein bands were visualized with Coomassie brilliant blue dye R-250. The BCA assay was done to determine the amount of protein in the membranes. We found that the BCA reagent reacted with the diacetylenic phosphatidylcholine polymer producing inaccurate results. This problem was avoided by first extracting the protein with

0.2% Triton X-100 for 2 h at room temperature. The diacetylenic polymer was then removed by centrifugation in a microfuge (Brinkmann, Westbury, NY). This procedure was shown by SDS-PAGE to extract essentially all the protein from the polymerized bilayer. Protein standard curves were carried out with known amounts of purple membrane. The phosphate assay of Bartlett [21] was used to determine the phospholipid content of the sample. Standard curves were made with known amounts of DMPC.

Apo-membrane was prepared by bleaching purple membrane in 0.5 M NH_2OH at pH 7.0 with light from a 150 watt slide projector filtered by an orange cut-off filter (Schott Glass Technologies, Inc., Duryea, PA). The retinal oxime was removed by repeated centrifugation with bovine serum albumin. The bR in polymerized membranes was bleached in an identical manner.

Freeze-fracture electron microscopy

Glycerol gradient purified membranes were used to freeze-fracture membrane/protein samples for examination in the transmission electron microscope (TEM). Samples which were in 20 to 30% glycerol (v/v) were taken directly from the glycerol gradient and used without further purification. Samples were processed by plunge freezing the 4°C vesicles in a nitrogen slush while in a cold room, and the room temperature lipids were processed in a propane jet freezer (RMC Inc., Tuscon, AZ) and the samples were fractured in a Balzers BAF400D apparatus (Balzers Inc. Liechtenstein). All samples were fractured without etching and the replica was formed from a 12.5 nm layer of platnium and a 12.5 nm layer of carbon. The shadow angle was at 45° to the replica plane. The replicas were cleaned by floating on a dilute acid or chlorox solution and then floating on distilled water and mounting on 200 mesh grids for examination in the Carl Zeiss EM-10C electron microscope, recorded on Kodak SO-163 film, and printed on polycontrast resin paper.

Results

Polymerization of $DC_{8,9}PC$ in mixed lipid membranes

Diacetylenes link to form a highly conjugated polymer containing alternate triple, double, and single bonds. The polymer backbone has a strong absorbance in the visible region of the spectrum [22]. The degree of polymerization in the membrane will be related to both the magnitude and wavelength of the visible absorbance peaks (see Discussion). The wavelength of the absorbance peak depends on the number of polymer units electronically coupled together. In general, the more polymer units coupled together the longer the wavelength of the absorbance peak. In addition, the magnitude of the absorbance in the visible region of the spectrum will be proportional to the concentration of

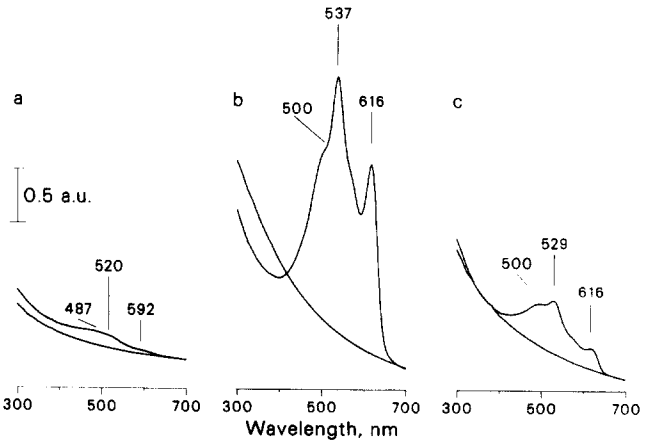


Fig. 1. Absorbance spectra of polymerized membranes at 4°C. The upper trace for each panel is the sample absorbance after 4 min of UV irradiation while the lower trace is before UV initiated polymerization. Each of the spectra in the figure corresponds to a $DC_{8,9}PC$ concentration of 0.36 mg/ml. (a) $DC_{8,9}PC$, (b) DNPC-$DC_{8,9}PC$, 2:1 mole ratio, (c) DMPC-$DC_{8,9}PC$, 2:1 mole ratio.

polymer units linked in polymer chains which are long enough to absorb in the visible region. Thus for a given set of conditions, if the relative amount and length of diacetylenic polymer in the bilayer increases this will produce increases in both the magnitude and wavelength of the visible absorbance peaks. Fig. 1 compares the visible absorbance spectra at 4°C of membranes composed of $DC_{8,9}PC$ (1a), 2:1 mole ratio DNPC-$DC_{8,9}PC$ (1b) and 2:1 mole ratio DMPC-$DC_{8,9}PC$ (1c) before and after 4 min of UV irradiation. The magnitude of the visible absorbance was significantly greater for the polymerized DNPC-$DC_{8,9}PC$ membranes than either the polymerized $DC_{8,9}PC$ membranes or the polymerized DMPC-$DC_{8,9}PC$ membranes. The spectra of polymerized DNPC-$DC_{8,9}PC$ and DMPC-$DC_{8,9}PC$ membranes contained several peaks and shoulders. The largest absorbance peaks for both of these mixed lipid systems were in the 530 to 535 nm range, while the longest wavelength absorbance peaks were near 616 nm. The absorbance peak for the polymerized $DC_{8,9}PC$ membranes was at 487 nm with only a relatively small absorbance shoulder near 590 nm. Therefore, we conclude that the polymerized DNPC-$DC_{8,9}PC$ membranes contained significantly more long polymer than polymerized $DC_{8,9}PC$ membranes, based on the qualitative arguments above and the data presented in Figs. 1–4.

Fig. 2 shows absorbance spectra the same membranes after they have been allowed to reach room temperature. The peaks at 616 nm in the spectra of the polymerized DNPC-$DC_{8,9}PC$ and DMPC-$DC_{8,9}PC$ membranes were not present. Two large absorbance peaks were observed for both polymerized membranes at approximately 530 nm and 500 nm. Allowing the polymerized $DC_{8,9}PC$ membranes to reach room tem-

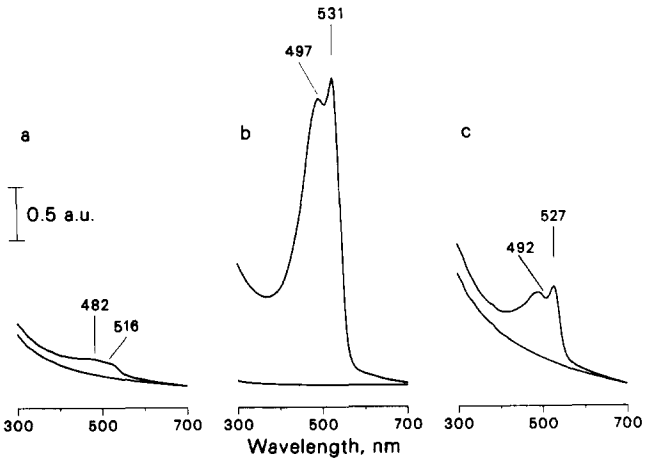


Fig. 2. Absorbance spectra of polymerized membranes at 24° C. These records are the same samples in Fig. 1 after they were allowed to reach 24° C. (a) $DC_{8,9}PC$, (b) DNPC-$DC_{8,9}PC$, 2:1 mole ratio, (c) DMPC-$DC_{8,9}PC$, 2:1 mole ratio.

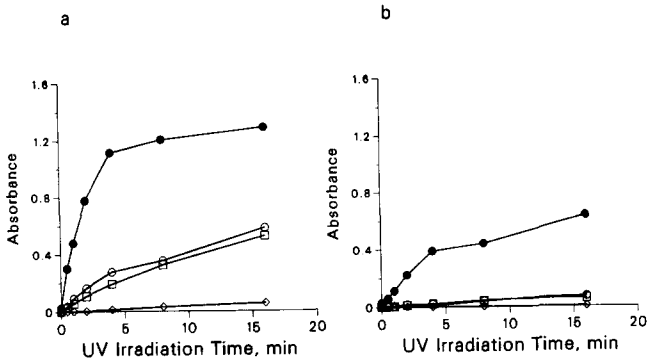


Fig. 4. Time course of UV initiated polymerization reaction of membranes. (a) The absorbance change at the indicated wavelengths as a function of polymerization time for the following membranes; $DC_{8,9}PC$ (450 nm, ◇), DPPC-$DC_{8,9}PC$ (460 nm, □), DMPC-$DC_{8,9}PC$ (460 nm, ○), and DNPC-$DC_{8,9}PC$ (470 nm, ●). The polymerized membranes were dissolved in chloroform/methanol/water. (b) The 535 nm absorbance of the polymer component removed by filtration through a 0.45 μm pore size filter as a function of polymerization time for the same membrane preparations.

perature had a much smaller effect on the spectra. The largest absorbance peak blue-shifted from around 490 nm to near 480 nm. The small 590 nm absorbance shoulder observed at 4° C was also reduced.

The polymerized membranes were dissolved in chloroform/methanol/water (65:25:4, v/v/v) and the absorbance spectra of the polymerized membranes dissolved in this solvent were recorded (Fig. 3). The lower trace for each record set is the absorbance spectra of the membranes prior to polymerization. The absorbance scale for the $DC_{8,9}PC$ and DMPC-$DC_{8,9}PC$ membranes is expanded 2-fold in order to reveal the fine features of the spectra. The absorbance peaks which are due to the polydiacetylene backbone of the polymerized DNPC-

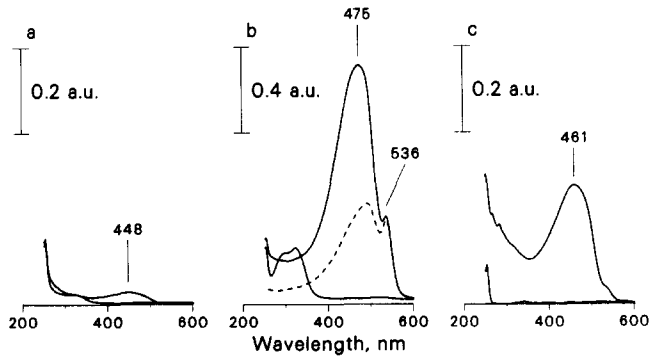


Fig. 3. UV-visible absorbance spectra of polymerized membranes dissolved in chloroform/methanol/water (65:25:4, v/v/v). The spectra before and after 4 min of polymerization are shown for each case. Each of the spectra in the figure corresponds to a $DC_{8,9}PC$ concentration of 0.24 mg/ml. The absorbance scales for the $DC_{8,9}PC$ and the DMPC-$DC_{8,9}PC$ spectra are expanded 2-fold in order to better show the smaller features of the spectra. The spectrum of the diacetylenic polymer component removed by filtration through a 0.45 μm pore size filter is shown by the dashed line for the DNPC-$DC_{8,9}PC$ polymerized membranes. (a) $DC_{8,9}PC$, (b) DNPC-$DC_{8,9}PC$, 2:1 mole ratio, and (c) DMPC-$DC_{8,9}PC$, 2:1 mole ratio.

$DC_{8,9}PC$ and DMPC-$DC_{8,9}PC$ membranes were at 475 and 461 nm, respectively, while the absorbance peak due to the polymerized $DC_{8,9}PC$ membranes was at 448 nm. The magnitude of the absorbance for the polymerized DNPC-$DC_{8,9}PC$ membranes was over 40 times that of the polymerized $DC_{8,9}PC$ membranes. The polymerized DNPC-$DC_{8,9}PC$ membranes also had an absorbance peak at about 535 nm, which was completely removed by filtration through a 0.45 μ pore size filter. The dashed line with the DNPC-$DC_{8,9}PC$ spectra (Fig. 3b) shows the absorbance spectrum of the polymer component removed by filtration. The spectrum was calculated by subtracting the polymer spectrum after filtration from the spectrum of before filtration. Some polymerization of $DC_{8,9}PC$ occurred in the $DC_{8,9}PC$ and DNPC-$DC_{8,9}PC$ bilayers even before the UV irradiation. This is indicated by the near-UV absorbance peaks observed in the spectra of $DC_{8,9}PC$ (Fig. 3a) and DPPC-$DC_{8,9}PC$ (Fig. 3b) membranes that were not yet polymerized with UV irradiation. These peaks were not due to impurities in the $DC_{8,9}PC$ or DNPC, since these lipids dissolved directly into this solvent did not have these absorbance peaks.

The time course of the UV-initiated polymerization is shown in Fig. 4. The absorbance of the largest absorbance peak in the spectra as a function of polymerization time is plotted in Fig. 4a for polymerized $DC_{8,9}PC$, DPPC-$DC_{8,9}PC$, DMPC-$DC_{8,9}PC$, and DNPC-$DC_{8,9}PC$ membranes dissolved in chloroform/methanol/water. The absorbance at 535 nm of the polymer component removed by filtration, as a function of polymerization time, is shown in Fig. 4b. As shown in Fig. 4b, after 16 min of exposure, the 535 nm absorbance for polymerized DNPC-$DC_{8,9}PC$ membrane was over 150-fold greater than the 535 nm absorbance

for the polymerized $DC_{8,9}PC$ membranes. The characteristics of the 535 nm absorbance peak from the polymerized DNPC-$DC_{8,9}PC$ membranes is further evidence that these polymerized membranes contain more long polymer than polymerized $DC_{8,9}PC$ membranes.

Protein insertion into the polymerized bilayers

Octyl glucopyranoside-solubilized bR was inserted the polymerized diacetylenic phosphatidylcholine membranes by overnight incubation (see Experimental procedures). Control polymerized membranes without bR were prepared in an identical manner. Fig. 5 compares the absorbance at 530 nm for gradient fractions collected from six different samples. The gradient distributions for the control polymerized membranes, i.e., no bR added, are shown by the open circle dashed line plot, while the solid circle solid line plots indicates the gradient distributions of the polymerized membranes incubated with bR. The gradient distributions for DNPC-$DC_{8,9}PC$ membranes incubated at 4°C are shown in Fig. 5a. Polymerized DNPC-$DC_{8,9}PC$ membranes incubated at 4°C without bR formed a very narrow band at density gradient fractions 7 and 8, while polymerized DNPC-$DC_{8,9}PC$ membranes incubated with the bR were significantly more dense, banding at fractions 3, 4 and 5. The density shift of the polymerized membranes shown in Fig. 5a must be due to close association of bR to the polymerized DNPC-$DC_{8,9}PC$ membranes, since all the other sample preparation factors were identical. When glycerol density gradients were run with purple membrane fragments all the membranes were found in the pellet. Bacteriorhodopsin incorporation into the polymerized membranes purified

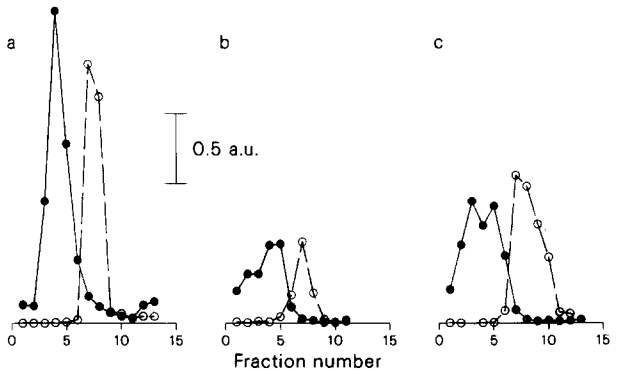


Fig. 5. Glycerol density gradients of polymerized membranes. Polymerized membrane samples were layered on 10–60% glycerol density gradient and centrifuged at 34000 rpm. The bottom of the gradient (60% glycerol) is labeled fraction 1. The solid circles solid line plots (●———●) show the absorbance at 530 nm for polymerized membranes incubated with bR, while the open circle dashed line plots (○———○) show the absorbance at 530 nm for control polymerized membranes. (a) DNPC-$DC_{8,9}PC$ incubated at 4°C, (b) $DC_{8,9}PC$ incubated at 4°C, (c) DNPC-$DC_{8,9}PC$ incubated at room temperature.

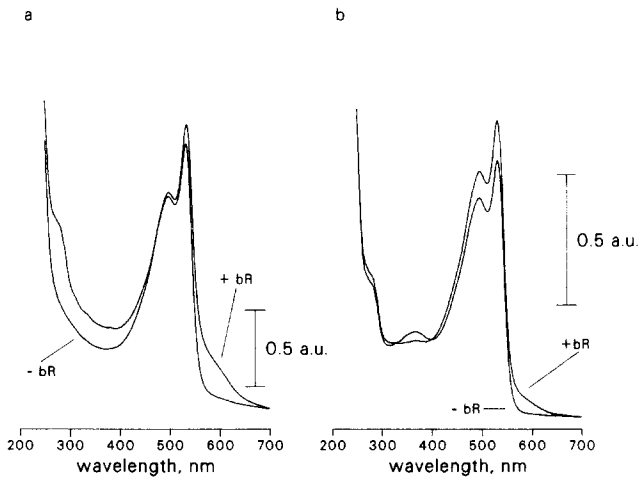


Fig. 6. Absorbance spectra of polymerized DNPC-$DC_{8,9}PC$ membranes. (a) The UV/visible absorbance spectra of polymerized DNPC-$DC_{8,9}PC$ membranes incubated with (+bR) and without (−bR) detergent solubilized bR. (b) Absorbance spectra of bR incorporated polymerized membranes before (+bR) and after (−bR) bleaching with 0.5 M NH_2OH.

by density gradient centrifugation was demonstrated by SDS-PAGE. Polymerized bR-DNPC-$DC_{8,9}PC$ membranes had a single clear protein band with the same molecular weight of bR (data not shown). The lipid to protein weight ratio for the polymerized bR-DNPC-$DC_{8,9}PC$ membranes was 8.4 ± 2.6 ($n = 7$) (see Experimental procedures). Thus polymerized bR-DNPC-$DC_{8,9}PC$ membranes were $11 \pm 4\%$ protein by weight, which corresponds to a lipid to protein mole ratio of about 300 to 1.

Fig. 6a compares the absorbance spectra of polymerized DNPC-$DC_{8,9}PC$ membranes incubated at 4°C with (+bR) and without bR (−bR). The spectra were recorded directly from fractions of the glycerol density gradient which were allowed to warm to room temperature. Both spectra contain significant amounts of polymerized membranes as indicated by the large absorbance peaks at 530 and 500 nm. The polymerized DNPC-$DC_{8,9}PC$ membranes incubated with bR also had two clear absorbance shoulders near 570 and 280 nm which indicated that these polymerized membranes were associated with bR. The presence of the 570 nm absorbance peak from bR, which results from a highly specific protein-chromophore interaction, is a good indication that the protein conformation was close to the native conformation. The 570 nm absorbance shoulder was not due to diacetylene polymer because this feature was eliminated following illumination of the membranes with orange light in the presence of 0.5 M NH_2OH (pH 7.0) as shown in Fig. 6b. The new absorbance peak near 360 nm was due to the absorbance of retinal which leaves the protein binding site after it was converted to retinal oxime by NH_2OH [19]. The 570 nm absorbance peak is a good indication that the

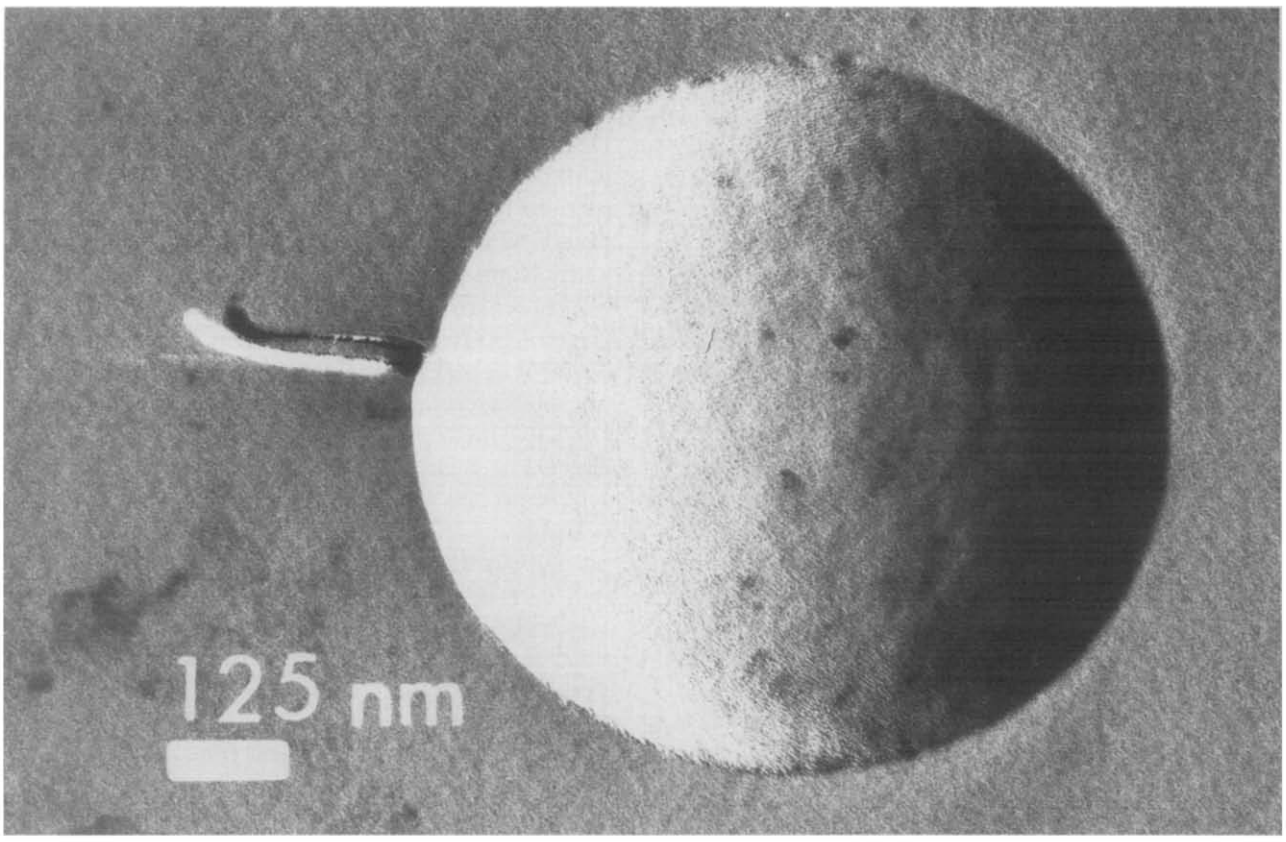
bR was functional, although there is a very high probability that the photocycle and proton pumping kinetics have been perturbed.

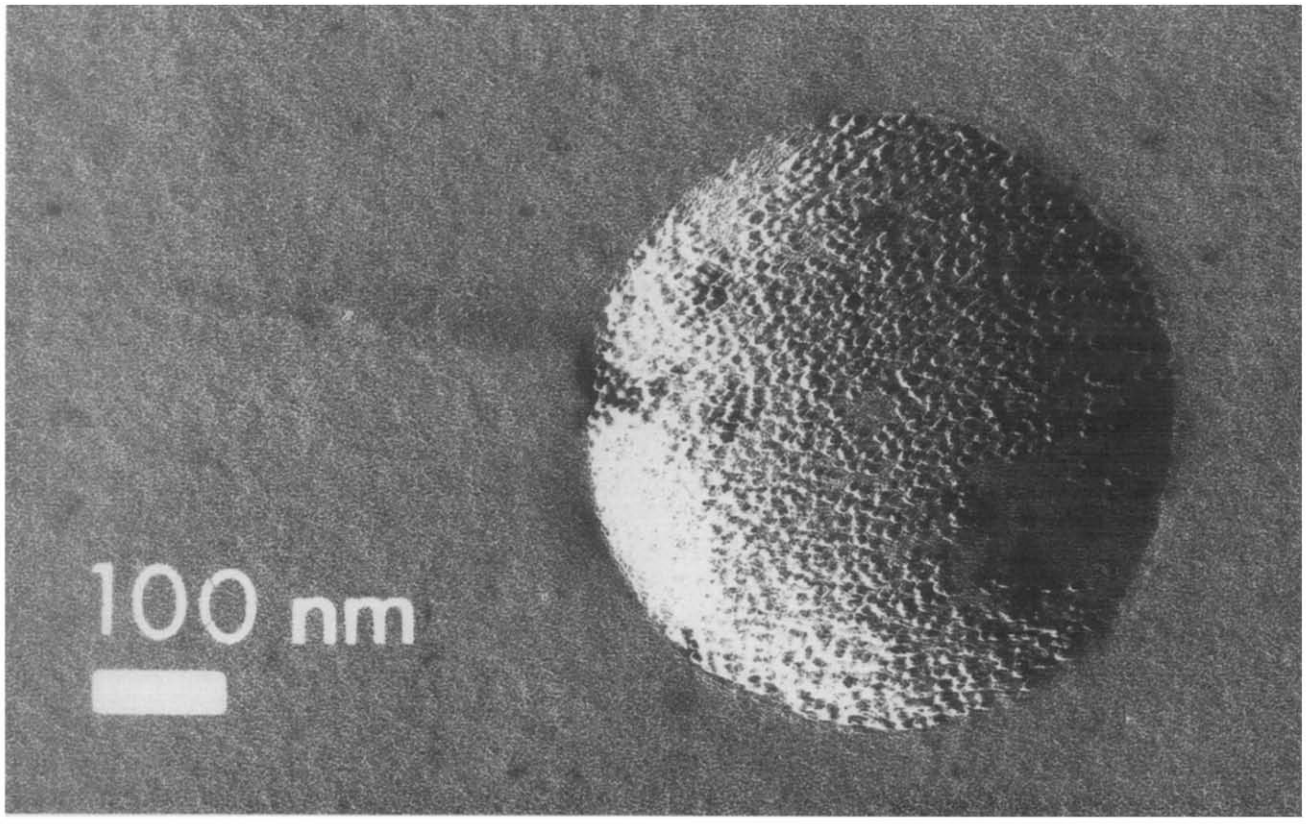
Fig. 5b shows control and bR containing density gradient fraction distributions of polymerized $DC_{8,9}PC$ membranes. The intensity of the 530 nm absorbance for these gradient fractions was 2–3-fold less than the polymerized DNPC-$DC_{8,9}PC$ membranes. The control polymerized $DC_{8,9}PC$ membranes were in approximately the same region of the density gradient as the control DNPC-$DC_{8,9}PC$ membranes (Fig. 5a). The polymerized $DC_{8,9}PC$ membranes incubated with bR were more dense than the control membranes. However, unlike the polymerized DNPC-$DC_{8,9}PC$ membranes, the polymerized $DC_{8,9}PC$ membranes incubated with bR did not form a single clear band with homogeneous color. In addition, density gradients of polymerized $DC_{8,9}PC$ membranes had a purple pellet at the bottom of the centrifuge tube. The UV/visible spectra of gradient fractions 4 and 5 indicated they contained most of the diacetylenic polymer with some bR. However, phosphate assays on these fractions revealed over a 10-fold decrease in the concentration of phospholipid in these fractions compared to the corresponding fractions for the polymerized DNPC-$DC_{8,9}PC$ membranes. The phosphate concentrations for the fractions 4 and 5 of the bR containing gradient shown in Fig. 5a were 52 and 11 μg/ml, respectively. In contrast, the phosphate concentration for the corresponding gradient fractions 4 and 5 of Fig. 5b were both less that 1 μg/ml. This indicated a significant amount of the $DC_{8,9}PC$ was lost during the reconstitution procedure using polymerized $DC_{8,9}PC$ membranes. The spectra of the high density fractions 1, 2, and 3 and the pellet revealed this material to be almost completely bR with relatively little diacetylenic polymer. The spectra of these high density membranes were similar to that of purple membrane sheets. The relative amount of protein in each fraction was estimated by the magnitude of the absorbance shoulder at 570 nm which is due primarily to bR. The density gradient fractions 4 and 5, which contained the largest fraction of the diacetylenic polymer, had only $19 \pm 12\%$ ($n = 5$) of the recovered bR associated with the polymerized $DC_{8,9}PC$ membranes. In contrast, $78 \pm 6\%$ ($n = 4$) of the recovered bR in the polymerized DNPC-$DC_{8,9}PC$ membranes was associated with the gradient fractions containing the diacetylenic polymer.

Fig. 5c shows a glycerol density gradients for polymerized DNPC-$DC_{8,9}PC$ membranes where the bR insertion procedure was done at room temperature. The density gradient distribution of control DNPC-$DC_{8,9}PC$ polymerized membranes incubated at room temperature was wider than the corresponding distribution for the polymerized membranes incubated at 4°C. The control polymerized DNPC-$DC_{8,9}PC$ membranes that were incubated at 4°C were blue-colored and formed very narrow dense bands (Fig. 5a). In contrast, the control polymerized membranes incubated at room temperature were orange and formed much broader bands (Fig. 5c). This density change might be due to the removal of DNPC and/or $DC_{8,9}PC$ from the bilayer by detergent during the room temperature dialysis. The solid line plot for Fig. 5c shows the distribution for the polymerized DNPC-$DC_{8,9}PC$ membranes that were incubated with bR and detergent at room temperature. A high and a low density membrane band were observed on the glycerol gradient. The high density band at fractions 1, 2 and 3 contained membranes with spectra similar to purple membrane as was the case for polymerized $DC_{8,9}PC$ membranes incubated with bR at 4°C. The less dense bands 4 and 5 contained most of the diacetylenic polymer. Similar to the experiments with the polymerized $DC_{8,9}PC$ membranes at 4°C, the phospholipid concentration for these fractions was several-fold less than for the corresponding fractions of polymerized DNPC-$DC_{8,9}PC$ membranes incubated at 4°C. The relative amount of bR recovered in the high and low density membrane bands varied widely for different experiments. The percent of recovered bR associated with the diacetylenic polymer fractions 4 and 5 was $50 \pm 30\%$ ($n = 4$).

Freeze-fracture electron microscopy was used to examine the size and morphology of the polymerized membranes and to confirm the presence of the protein in the bilayer. The control polymerized DNPC-$DC_{8,9}PC$ membranes were vesicles 0.2 to 0.8 μm in diameter *. A typical micrograph of replicas of control DNPC-$DC_{8,9}PC$ polymerized membranes is shown in Fig. 7a. The fracture planes through the membrane of the control replicas were always smooth. The DNPC-$DC_{8,9}PC$ polymerized membranes that were incubated with bR were also vesicles 0.2 to 0.8 μm in diameter. Typical micrographs of replicas of these polymerized membranes are shown in Figs. 7b and 7c. The fracture plane was through the polymerized bilayer for Fig. 7b, while in Fig. 7c the fracture plane cut across the polymerized vesicle. The polymerized DNPC-$DC_{8,9}PC$ membranes incubated with bR were covered with intramembranous particles which were 70 to 100 Å in diameter. This suggests that each particle was composed of a bR trimer, rather than a single monomer [23]. The replica

* The average diameter of the DNPC-$DC_{8,9}PC$ vesicles before polymerization at 4°C was also in the 0.2 to 0.8 μm range (Price, R., Singh, A. and Ahl, P., unpublished data). No significant change in the diameter or shape of the vesicles was observed after polymerization. However, there did appear to be a significantly larger fraction of small vesicles in the 0.2 to 0.3 μm range before the octyl glucopyranoside incubation. This suggests that some vesicle fusion may be occurring during the protein insertion step.


125 nm


100 nm

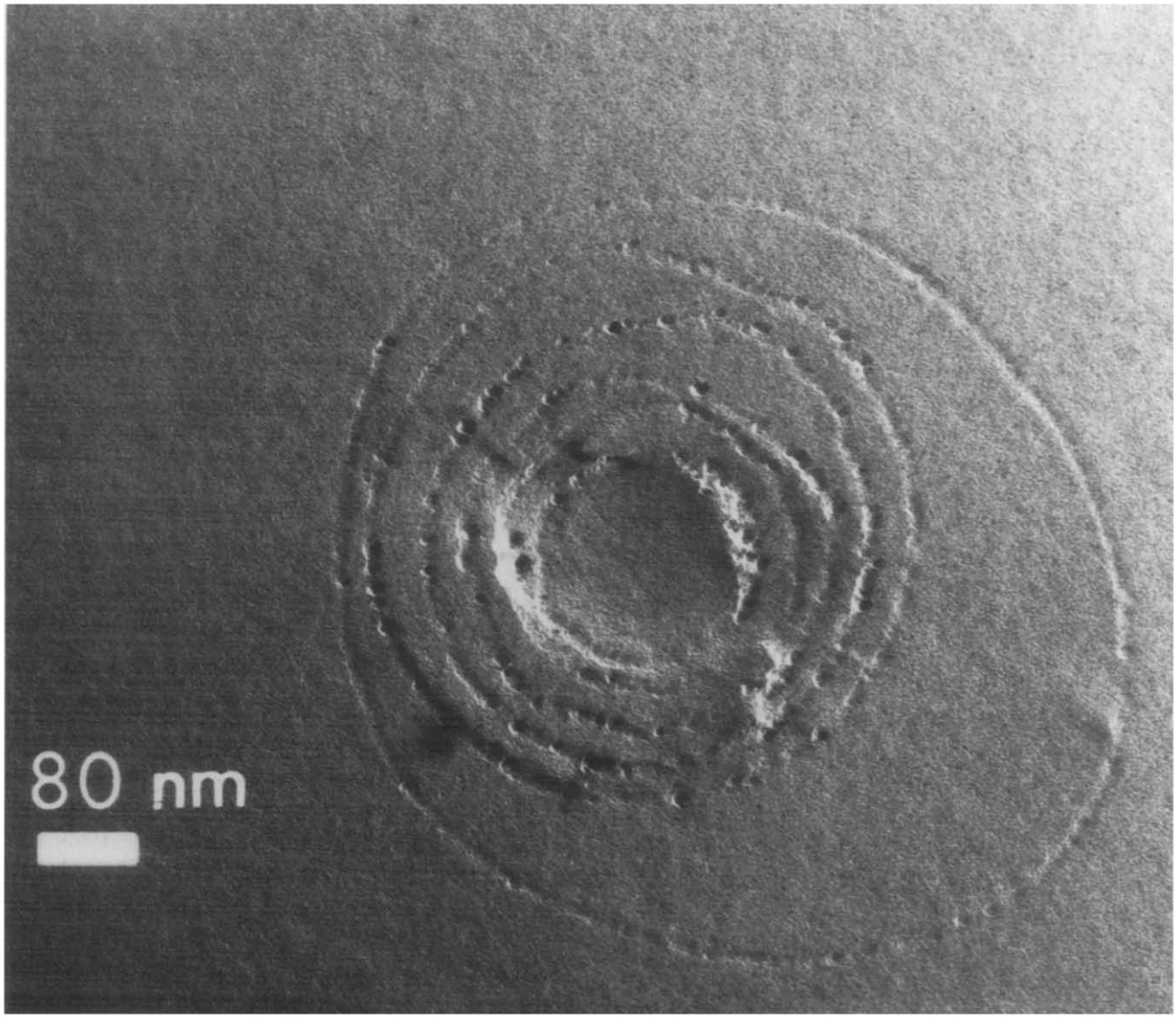


Fig. 7. Freeze-fracture electron micrographs: (a) control polymerized DNPC-$DC_{8,9}$PC membranes, i.e., incubated without bR, (b and c) polymerized DNPC-$DC_{8,9}$PC membranes incubated with bR. The micrographs shown are of DNPC-$DC_{8,9}$PC polymerized membranes that were rapidly frozen in liquid N_2 from 4°C, i.e., blue-colored membranes. Similar intramembranous particles were observed with polymerized DNPC-$DC_{8,9}$PC membranes that were allowed to reach room temperature before they were rapidly frozen in liquid N_2, i.e., orange-colored membranes.

shown in Fig. 7c suggests some of the DNPC-$DC_{8,9}$PC vesicles were multilamellar.

Discussion

Polymerization of $DC_{8,9}PC$ in mixed lipid membranes

Diacetylenes cross-link via a 1,4-addition reaction to form a highly conjugated linear polymer containing alternating triple, double, and single bonds. The polydiacetylene structure has been rigorously established for polymerized diacetylenic model compounds by X-ray analysis [24]. Other possible chromophore groups, such as aromatic rings, which could, in principle, be formed via a 1,2-addition reaction have not been observed. Since there appears to be a close analogy between the spectroscopy of polydiacetylenes and polyenes [25], the modified free-electron theory of Kuhn [26] can be used to calculate the effective conjugation of the polymer. Using this theory, a lower bound for the length of the individual polydiacetylene chains can be determined from the UV/visible absorbance spectrum. The effective polymer conjugation is the average number of electronically coupled polymer units and can be calculated from the wavelength of the absorbance peaks in the spectra. The effective conjugation is a lower bound for the total number of units in a polymer chain since polymer segments may be electronically decoupled from each other by twists or bends. The wavelength of an

absorbance peak is inversely related to the optical bandgap energy between the ground and excited state. The optical bandgap energy, E, in eV is related to the number of multiple bonds, N, in the electronic conjugation by the following equation:

$$E = V_0 + \left(\frac{h^2}{4mL_0^2} - \frac{V_0}{4}\right)\left(\frac{1}{N+1/2}\right) \quad (1)$$

where h = Planck's constant, m = mass of electron, L_0 = length of a unit of conjugation, V_0 = optical band gap in eV for an infinite conjugation number.

Model compound studies indicate V_0 is about 2.0 eV for both polyenes and polydiacetylenes in a nonpolar environment like the interior of a lipid bilayer [27]. This value for V_0 corresponds to an absorbance peak at 620 nm. The effective conjugation for a polydiacetylene is $N/2$ since there are two multiple bonds per polymer units. The physical length of the effective conjugation, i.e., the effective conjugation length, can be calculated using polymer bond distances obtained from the structures of polymerized crystals of diacetylenic molecules [27]. The effective conjugation of the polydiacetylene can also be determined with approximately the same accuracy with other spectroscopic techniques that are sensitive to electron delocalization in the polymer backbone such as ^{13}C-NMR and Raman spectroscopies [28].

Table I shows for each of the major absorbance features labeled in Figs. 1 and 2, the relative intensity of the absorbance (A_{rel}), the effective conjugation (C_{eff}), and the length of the effective conjugation measured in nanometers (L_{eff}). This table clearly illustrates the large effect of the presence of DNPC in the bilayer has on the polymerization reaction. The effective conjugation for the largest peak in the spectra of the polymerized $DC_{8,9}PC$ membranes at 4°C was only eight polymer units (487 nm), while the effective conjugation for the relatively small shoulder at 592 nm was 30 units. In contrast, the relatively large 616 nm absorbance peak observed for polymerized DNPC-$DC_{8,9}PC$ membranes at 4°C corresponds to an effective conjugation of over 300 polymer units. As indicated in Table I, this corresponds to an effective conjugation length of over 0.3 μm for this DNPC-$DC_{8,9}PC$ polymer fraction. This value is a lower bound for the polymer chain length. When the polymerized DNPC-$DC_{8,9}PC$ membranes were allowed to reach room temperature the absorbance peaks irreversibly blue-shifted to 531 and 497 nm which correspond to an effective conjugation of about 10 units. Since allowing the polymerized membranes to reach room temperature would not break covalent bonds, this reduction in the effective conjugation must result from twists or bends of polymer in the DNPC-$DC_{8,9}PC$ bilayer. Although the effective conujugation of the polymer has changed as result of twist or bends in the polymer, the number of polymer units in the polymer chain must be the same.

The absorbance of the diacetylenic polymer dissolved in chloroform/methanol/water was blue-shifted compared to the spectra of polymer in the polymerized bilayers. The absorbance peaks for the polymerized DNPC-$DC_{8,9}PC$ and $DC_{8,9}PC$ membranes dissolved in this solvent were at 471 and 448 nm, respectively, which corresponds to an effective conjugation length of only 5 to 7 units for both polymers. However, the magnitude of the peak absorbance for the polymerized DNPC-

TABLE I

Effective conjugation of polymer in the bilayer [a]

Membrane	4°C				24°C			
	λ [b]	A_{rel} [c]	C_{eff} [d]	L_{eff} [e]	λ	A_{rel}	C_{eff}	L_{eff}
DNPC-$DC_{8,9}PC$	616	0.31	340	370	531 (3.04)	0.52	13	14
	537 (2.36)	0.42	14	15	497	0.48	9	10
	500_{sh} [f]	0.27	9	10				
DMPC-$DC_{8,9}PC$	616	0.14	340	370	527 (0.745)	0.54	12	13
	529 (0.42)	0.48	13	14	492	0.46	8	9
	500_{sh}	0.38	9	10				
$DC_{8,9}PC$	592_{sh}	0.20	31	50	516_{sh}	0.49	10	11
	520_{sh}	0.38	11	12	482 (0.269)	0.51	7	8
	487 (0.17)	0.42	8	9				

[a] The absorbance peaks and shoulders are from the spectra shown in Figs. 1 (4°C) and 2 (24°C).

[b] λ, the wavelength in nanometers of the absorbance peak or shoulder. The absorbance of the largest peak in the spectra is shown in parentheses.

[c] A_{rel}, the relative absorbance of the peak or shoulder. This parameter was calculated by dividing the absorbance at the peak or shoulder by the sum of the absorbances at all the peaks or shoulders in the spectrum.

[d] C_{eff}, the effective conjugation of the absorbance peak or shoulder. This parameter was calculated using Eqn. 1.

[e] L_{eff}, the effective conjugation length in nanometers of the absorbance peak or shoulder. The diacetylenic bond distances are from Ref. 27.

[f] $_{sh}$ indicates absorbance shoulder.

$DC_{8,9}PC$ membranes was over 40 times that of the polymerized $DC_{8,9}PC$ membranes. This clearly indicates that DNPC improved the polymerization efficiency of $DC_{8,9}PC$ *. Although the effective conjugation of the diacetylenic polymer from the polymerized DNPC-$DC_{8,9}PC$ membranes was much shorter dissolved in chloroform/methanol/water, it is very unlikely that the number of units in the polymer changed. Instead, the reduction in the effective conjugation indicates that the diacetylenic polymer became bent or twisted enough in this solvent to disrupt the multiple bond conjugation. The behavior of the 536 nm peak in the DNPC-$DC_{8,9}PC$ spectra is a clear indication that this was occurring (Fig. 3b). Although the conjugation length for this peak was only 15 nm the dimensions of polymer chain must be significantly larger. As shown in Fig. 3b, this absorbance peak was removed by filtration through 0.45 μm pores. This 536 nm absorbance peak in organic solvents was probably due to the polymer component that corresponds to the 616 nm absorbance peak observed in the polymerized DNPC-$DC_{8,9}PC$ membranes at 4°C after the polymer has become bent or twisted. The behavior of this relatively large absorbance peak indicates that the polymerized DNPC-$DC_{8,9}PC$ membranes had a significant fraction of long chain polymer. In contrast, as shown in Fig. 4b, no significant amount polymer was removed from the polymerized $DC_{8,9}PC$ membranes samples by filtration. Apparently, extremely long chain length polymer was never present in the polymerized $DC_{8,9}PC$ membranes.

How does the non-polymerizable phosphatidylcholine DNPC produce such a large improvement the polymerization efficiency of $DC_{8,9}PC$? The UV-initiated polymerization of diacetylene containing molecules is very efficient when the diacetylene bonds are closely packed and well ordered. For example, polymerization of multilayers films of diacetylenic fatty acids assembled by the Langmuir-Blodgett technique produced significant amounts of blue-colored polymer [29]. However, bilayers of diacetylenic phosphatidylcholine, such as $DC_{8,9}PC$, did not polymerize as extensively [30,31,15]. The DNPC must somehow alter the packing of $DC_{8,9}PC$ in the bilayer to position the diacetylenic groups in a better orientation for efficient polymerication. The most direct mechanism for DNPC to affect $DC_{8,9}PC$ packing would be for DNPC to intermix with $DC_{8,9}PC$ in the bilayer to form a mixed lipid phase. It is more difficult to imagine how DNPC could alter the packing of $DC_{8,9}PC$ if the two lipids were phase separated in the bilayer. Preliminary differential scanning calorimetry of DNP-$DC_{8,9}PC$ mixture indicated no phase separation (Singh, A. and Gaber, B.P., unpublished data). Lopez, et al. [32] examined the UV-initiated polymerization of $DC_{8,9}PC$ mixed at a 1:2 mole ratio with either distearoylphosphatidylcholine (DSPC) or dioleoylphosphatidylcholine (DOPC). In both cases, the two phospholipids were found to phase separated by differential scanning calorimetry at the temperatures where the polymerization was done. In contrast to the effect of DNPC, these long acyl chain phosphatidylcholines had no effect on efficiency of UV initiated polymerization of $DC_{8,9}PC$.

How is the packing of $DC_{8,9}PC$ in the bilayer altered by DNPC to improve the polymerization efficiency? Recent low angle X-ray diffraction measurements and model building indicate that in pure $DC_{8,9}PC$ bilayers the α- and β-diacetylene groups are displaced relative to each other by about 6 Å [33]. The DNPC might alter the conformation of $DC_{8,9}PC$ in such a manner to move the two diacetylene groups closer together. This would produce tighter packing of the diacetylene groups which could lead to improved polymerization efficiency. Experiments with diacetylenic dimethyldialkylammonium amphiphiles in which the two diacetylenic groups must penetrate to the same depth in the bilayer support this hypothesis. Liposomes formed from diacetylenic dimethyldialylammonium amphiphiles polymerize very efficiently forming dark blue polymers after a few seconds of UV irradiation [32]. Another possibility is that the DNPC might induce the distal methylene segments of $DC_{8,9}PC$ to interdigitate with those of the opposite monolayer. Recent low angle X-ray diffraction measurements on DNPC-$DC_{8,9}PC$ membranes, however, showed a bilayer thickness of about 69 Å which indicated that $DC_{8,9}PC$ interdigitation did not occur [34].

Bacteriorhodopsin incorporation

Our results indicate that under certain conditions extensive polymerization of diacetylenic phospholipid bilayers did not block efficient membrane protein insertion. Although polymerized DNPC-$DC_{8,9}PC$ membranes contained a relatively large amount of polymer there is still enough space between the polymer chains

* The percentage of $DC_{8,9}PC$ converted to polymer can be estimated from the extinction coefficient for other diacetylenic polymers dissolved in similar organic solvents. The extinction coefficient of a completely polymerized dodeca-5,7-diyn-1,12-ylene-di-*p*-toluenesulfonate dissolved in chloroform/methanol is $16.7 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$ per polymer unit at 470 nm (Wenz and Wegner (1982) Makromol. Chem., Rapid Commun. 3, 231–237). This value is probably a reasonable estimate for the extinction coefficient per polymer unit of $DC_{8,9}PC$ polymer dissolved chloroform/methanol/water. We used this value to get a lower estimate of the percentage of $DC_{8,9}PC$ molecules that were in polymer chains of 6 to 7 units or longer. The estimates calculated for the polymer spectra shown in Fig. 3 are: 0.5% for $DC_{8,9}PC$, 7% for DMPC-$DC_{8,9}PC$, and 25% for DNPC-$DC_{8,9}PC$ polymerized membranes. The polymer estimate for the polymerized DNPC-$DC_{8,9}PC$ membranes is probably too low because it does not account for the polymer corresponding to the 536 nm peak. The $DC_{8,9}PC$ cross-linked to form dimers and trimers is not included in these estimates, since these molecules would not have significant absorbance at 470 nm.

for insertion of the membrane proteins. This suggests that there was not a large amount of inter-chain polymer cross-linking in these membranes. The relatively large amount of DNPC in the bilayer probably had an important role in separating the polymer chains and/or preventing inter-chain polymer cross-linking. Thus, polymerized DNPC-$DC_{8,9}PC$ membranes were not an impenetrable shell and the polymer did not appear to have an extensive amount of inter-chain cross-links.

Bacteriorhodopsin was more efficiently inserted into polymerized DNPC-$DC_{8,9}PC$ membranes than into polymerized $DC_{8,9}PC$ membranes. In addition, when polymerized $DC_{8,9}PC$ membranes were used, membranes quite similar to the purple membrane in composition were formed in the reconstitution procedure. Both these observations are probably due to the instability of the polymerized $DC_{8,9}PC$ membranes to the detergent dialysis step. As discussed in Results, there was a large decrease in the amount of phospholipid found in the polymerized $DC_{8,9}PC$ samples that had been incubated with the detergent. The detergent solubilized bR contained a small amount of endogenous *H. halobium* lipids. This lipid component was less than 7% of the total lipid in the reconstitution mixture. A competition must exist during the reconstitution reaction between the bR insertion into the polymerized membrane and reformation of the purple membrane with the endogenous lipids. Since large amounts of $DC_{8,9}PC$ appeared to be lost during dialysis of polymerized $DC_{8,9}PC$ membranes, protein insertion into the polymerized membranes must have become inefficient relative to reformation of the purple membrane fragments with endogenous *H. halobium* lipid. Polymerized DNPC-$DC_{8,9}PC$ membranes seem to be more stable to octyl-glucopyranoside at 4°C, since phospholipid was not lost during the detergent incubation. The increased stability of polymerized DNPC-$DC_{8,9}PC$ membranes to detergent 4°C was probably the principal reason for the improved protein insertion efficiency. The reduced efficiency for protein incorporation into polymerized DNPC-$DC_{8,9}PC$ membranes at room temperature probably results from a similar process.

O'Brien and co-workers [14] reported the insertion of octyl-glucopyranoside solubilized rhodopsin into polymerized membranes composed of 1:1 mole ratio of the polymerizable lipid DENPC and the non-polymerizable lipid DOPC. Although our reconstitution method is similar to the procedure used by these authors the characteristics of polymerized DOPC-DENPC membranes were very different. The dienoyl polymer formed by polymerization of DENPC is not highly conjugated like polydiacetylene. These authors reported that the detergent solubilized rhodopsin appeared to be inserted into domains of DOPC in the membrane. As discussed above, we believed the co-existence of separate domains of DNPC and $DC_{8,9}PC$ was unlikely at the temperature at which the most efficient protein insertion occurs. Therefore, an insertion mechanism similar to the mechanism observed by O'Brien and co-workers was probably not occurring for bR insertion into the polymerized DNPC-$DC_{8,9}PC$ membranes. The protein was probably inserted into the space between the individual polymer chains in the bilayer which were separated from each other by DNPC molecules.

Conclusion

We have demonstrated that the integral membrane protein bacteriorhodopsin can be efficiently inserted into extensively polymerized diacetylenic phosphatidylcholine bilayers. The key feature of the reconstitution procedure was mixing the polymerized lipid with short acyl chain phosphatidylcholine at a 2:1 mole ratio to improve both diacetylene polymerization and protein insertion.

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References

1 Johnston, D.S., Sanghera, S., Pons, M. and Chapman, D. (1980) Biochim. Biophys. Acta 602, 57–69.
2 Hub, H.H., Hupfer, B., Koch, H. and Ringsdorf, H. (1980) Angew. Chem., Inst. Edn. Engl. 19, 938–940.
3 O'Brien, D.F., Whitsides, T.H. and Klingbiel, R.T. (1981) J. Polym. Sci., Polym. Lett. Edn. 19, 95–101.
4 Regen, S.L., Czech, B. and Singh, A. (1980) J. Am. Chem. Soc. 102, 6638–6639
5 Akimoto, A., Dorn, K., Gros, L., Ringsdorf, H. and Schupp, H. (1981) Angew. Chem., Int. Edn. Engl. 20, 90–91.
6 Gros, L., Ringsdorf, H. and Schupp, H. (1981) Angew. Chem., Int. Edn. Engl. 20, 305–325.
7 Dorn, K., Kingbiel, R.T., Specht, D.P., Tyminski, P.N., Ringsdorf, H. and O'Brien, D.F. (1984) J. Am. Chem. Soc. 106, 1627–1633.
8 Gaber, B.P., Schnur, J.M. and Chapman, D. (eds.) (1988) Biotechnological Applications of Lipid Microstructures, Plenum Press, New York.
9 Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 52, 587–616.
10 Wagner, N., Dose, K., Koch, H. and Ringsdorf, H. (1981) FEBS Lett. 132, 313–318.
11 Pabst, R., Ringsdorf, H., Koch, H. and Dose, K. (1983) FEBS Lett. 154, 5–9.
12 Yager, P. (1986) Biosensors 2, 363–373.
13 Tyminski, P.N., Latimer, L.H. and O'Brien, D.F. (1985) J. Am. Chem. Soc. 107, 7769–7770.
14 Tyminski, P.N., Latimer, L.H. and O'Brien, D.F. (1988) Biochemistry 27, 2696–2705.
15 O'Brien, D.F., Klingbiel, R.T., Specht, D.P. and Tyminski, P.N. (1986) Ann. N.Y. Acad. Sci. 446, 282–295.

16 Singh, A. and Gaber, B.P. (1988) in Applied Bioactive Polymeric Materials (Gebelein, C.G., Carraher, C.E. and Foster, V.R., eds.), pp. 239–249, Plenum Press, New York.
17 Becher, B. and Cassim, J.Y. (1975) Prep. Biochem. 5, 161–178.
18 Singh, A. (1990) J. Lipid Res. 31, 1522–1525.
19 Oesterhelt, D. and Stoeckenius, W. (1971) Nature (Lond.) 233, 149–151.
20 Laemmli, U.K. (1970) Nature (Lond.) 277, 680–685.
21 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
22 Wegner, G. (1979) in Molecular Metal (Hatfield, W.E., ed.), pp. 209–242, Plenum Press, New York.
23 Cherry, R.S., Muller, U., Henderson, R. and Heyn, M.P. (1978) J. Mol. Biol. 121, 283–298.
24 Aime, J.P., Schott, M., Bertault, M. and Toupet, L. (1988) Acta Cryst. B44, 617–624.
25 Baughman, R.H. and Chance R.R. (1976) J. Polym. Sci., Polym. Phys. Edn. 14, 2037–2045.
26 Kuhn, H. (1959) Fortschr. Chem. Org. Naturst. 17, 404.
27 Exarhos, G.J., Risen, W.M. and Baughman, R.H. (1976) J. Am. Chem. Soc. 98, 481–487.
28 Wenz, G., Müller, M.A., Schmidt, M. and Wegner, G. (1984) Macromolecules 17, 837–850.
29 Tieke, B., Lieser, G. and Wegner, G. (1979) J. Polym. Sci., Polym. Chem. Edn. 17, 1631–1644.
30 Lopez, E., O'Brien, D.F. and Whitesides, T.H. (1982) J. Am. Chem. Soc. 104, 305–307.
31 Johnston, D.S., McLean, L.R., Whittam, A., Clark, A.D. and Chapman, D. (1983) Biochemistry 22, 3194–3202.
32 Lopez, E., O'Brien, D.F. and Whitesides, T.H. (1982) Biochim. Biophys. Acta 693, 437–443.
33 Schoen, P.E., Yager, P., Sheridan, J.P., Price, R., Schnur, J.M., Singh, A., Rhodes, D.G. and Blencher, S.L. (1987) Mol. Cryst. Liq. Cryst. 153, 357–366.
34 Rhodes, D.G. and Singh, A. (1989) Biophys. J. 55, 138a.