EFFECTS OF PRESSURE AND TEMPERATURE ON THE M412 INTERMEDIATE OF THE BACTERIORHODOPSIN PHOTOCYCLE

Implications for the Phase Transition of the Purple Membrane

MOTOYUKI TSUDA, RAJNI GOVINDJEE, AND THOMAS G. EBREY
Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

ABSTRACT The effects of pressure and temperature on the decay kinetics of the M412 (M) intermediate in the photocycle of bacteriorhodopsin were studied to provide information about the phase transitions of the purple membrane lipids. The activation volume ($\Delta V\ddagger$) for the decay of M is expected to be different below and above a phase transition. However, no abrupt change in $\Delta V\ddagger$ was found from 3.5° to 60°C. But a sharp break was observed in a plot of the logarithm of the rate of M decay vs. pressure. Extrapolation of this break point to standard atmospheric pressure gives a temperature of −42°C, which probably corresponds to the phase transition of the purple membrane lipids. This conclusion is supported by studies of the effect of pressure on the M kinetics of bacteriorhodopsin incorporated into dimyristoylphosphatidylcholine vesicles, whose phase transition has previously been characterized.

INTRODUCTION

Bacteriorhodopsin, the only protein in the purple membrane of *Halobacterium halobium*, spans the lipid bilayer. Proton translocation from the inside to the outside of the cell membrane is initiated by light isomerizing the retinal chromophore of bacteriorhodopsin. The bacteriorhodopsin is immobilized as trimers in a two-dimensional hexagonal lattice, presumably by protein-protein interaction. Lipids, which account for 25% of the membranes by weight, fill the space between bacteriorhodopsin molecules. Little is known about the packing of the lipids. When the protein molecules associate into trimers and the lipid is distributed in the center and on the perimeter of the trimers, most of the available lipid is required to form a layer that is directly in contact with the protein. (For a review see references 1 and 2).

The phase transitions of the purple membrane lipids have been extensively studied but the results appear conflicting. Several research groups have observed a thermotropic transition of the purple membrane around 30°C, using such techniques as spin labels (3), proton NMR (4, 5), and kinetics of the phototransients (6, 7). Though Caplan and co-workers (6, 8) concluded that the changes around 30°C were due to a lipid-phase transition, several other groups failed to observe this transition. A differential

scanning calorimetry study by Jackson and Sturtevant (9) did not reveal any phase transition over the range from 0° to 75°C. They found a small endothermic transition at 80°C and a larger one at ~100°C. The 100°C transition corresponded to the irreversible denaturation of bacteriorhodopsin. The smaller 80°C transition was interpreted as a changing cooperativity of bacteriorhodopsin in the crystalline structure of the purple membrane. Hiraki et al. (10) also observed this 80°C transition using both x-ray diffraction and circular dichroic spectroscopy, but did not observe any other transition from −80° to 75°C in the purple membrane.

Phase transitions of the isolated lipids have also been studied. The main polar lipids of purple membranes have a saturated highly branched phytanyl moiety (3, 7, 10, 11, tetramethylhexadecyl), which would suggest that the phase transition temperature might be very low (11) or even absent (12). Chen et al. (13) found that membranes made from the total lipid extracted from purple membrane have a phase transition at −45°C. However, Hiraki et al. (10) failed to observe by x-ray diffraction a phase transition of the extracted lipid from purple membrane between −80° and 75°C.

A large volume change is expected in going from the liquid to the crystalline state of membranes (14). Thus, the magnitude of the molar volume change (ΔV) or the activation molar volume ($\Delta V\ddagger$) for a reaction of a membrane protein is expected to be different below and above the phase transition temperature, if the protein's confor-

___

Dr. Tsuda's present address is the Department of Physics, Sapporo Medical College, Sapporo, Japan.

mational change is dependent on the lipid environment (15, 16). Moreover, the phase-transition temperature shifts to higher temperature at high pressures, so that phase-transition temperatures below 0°C at atmospheric pressure can be observed around room temperature under high pressures (15, 16). In the present paper,we measured the effect of pressure and temperature on the decay of the M412 (M) intermediate of the bacteriorhodopsin photocycle. Our data suggest that the phase-transition temperature of the lipids of purple membrane is around −42°C. This method of determining the phase-transition temperature was checked by studies of phase transition of bacteriorhodopsin(BR) incorporated into dimyristoylphosphatidylcholine (DMPC) vesicles.

MATERIALS AND METHODS

Purple membrane (PM) was prepared from *Halobacterium halobium* S9 by the method of Becher and Cassim (17). DMPC was obtained from Sigma Chemical Co. (St. Louis, MO) and was used without further purification. Cholic acid from Sigma Chemical Co. was decolorized with activated charcoal and recrystallized twice from 50% ethanol. Imidazole was recrystallized from benzene.

Preparation of Bacteriorhodopsin-Dimyristoylphosphatidylcholine Vesicles (BR/DMPC Vesicles)

BR/DMPC vesicles were prepared following the method of Bakker and Caplan (18). 10 mg PM was solubilized in 3 ml 10% sodium cholate in 100 mM Tris-HCl buffer, 150 mM KCl. To partially substitute DMPC for the native lipids associated with BR, the solubilized BR was mixed with 25 mg of DMPC and incubated for 2 h at room temperature. This mixture was then layered on a linear 20–60% sucrose gradient and centrifuged for 16 h at 100,000 g. The purple band was collected, washed with 150 mM KCl, and the substitution procedure repeated with another 25 mg DMPC. After the final run on the sucrose gradient, the purple band was collected, washed with 150 mM KCl and dialyzed against 150 mM KCl for 24 h, during which the medium was replaced three times. These preparations still contained a substantial amount of endogenous PM lipid.

Flash Photolysis Under High Pressure

M kinetics were determined by measuring transmission changes at 410 nm following flash excitation with a kinetic spectrophotometer. A dye laser (model DL1000, Phase-R)with orange II dye was used to provide an actinic flash at 580 nm, half-pulse width of 0.5 μs. The sample was kept in a cylindrical quartz high-pressure cell (8 mm diam.). The monochromatic measuring beam transversed the sample at right angles to the actinic flash and was detected by a photomultiplier after passing through a second monochromator. The preamplified signal was digitized and accumulated by a signal averager. An average of four flashes was taken.

The apparatus used for pressure generation and the optical bomb are described in detail elsewhere (19, 20). The high-pressure optical bomb was constructed from stainless steel and rated to 3 k bar. The optical bomb had four windows; three were of synthetic sapphire and the fourth was for thermocouple electrodes. A PM sample was placed in the cylindrical internal cell made of quartz (6 mm i.d., 0.3 ml volume) and isolated from the pressure fluid by means of a quartz cylinder. All samples were buffered at pH 7.2 with 10 mM imidazole-HCl buffer; for this buffer the pH decreases by no more than 0.1 units on increasing the pressure from 1 atm to 6 k bar (19, 21). All samples were light adapted. Constant pressure at different temperatures in a high-pressure cell was

maintained with the use of an oil pump. The pressure of the sample was measured by a Heize gauge. Constant temperature was maintained by circulating a thermostatted fluid in the optical bomb.

Turbidity of DMPC vesicles and BR/DMPC vesicles was measured by transmittance change at 460 nm. Temperature of the sample was measured by a thermocouple inserted in the high pressure fluid. Transmittance (x-axis) and temperature (y-axis) were simultaneously displayed on an X-Y recorder.

RESULTS

Purple Membrane Sheets

A typical time course of the decay of the photointermediate M (1 bar) is shown in Fig. 1 a. An Arrhenius plot of the M decay shows an apparent break around 30°C as shown in Fig. 2. This value is consistent with other reports of a discontinuity in the properties of PM around 30°C observed using several different techniques (4–8). If the 30°C thermotropic transition is due to a liquid-crystalline transition of the lipids, the activation volume for the M decay is expected to be different below and above the phase-transition temperature. The activation volume $(\Delta V\ddagger)$ for the decay of M is obtained by measuring the effect of pressure on the rate constant k: $\Delta V\ddagger = -RT(\partial \ln k/\partial P)_T$.

Fig. 1 shows kinetic data for M decay at different pressures (1, 600, 1,500, and 2,000 bar), at 25°C. The rate
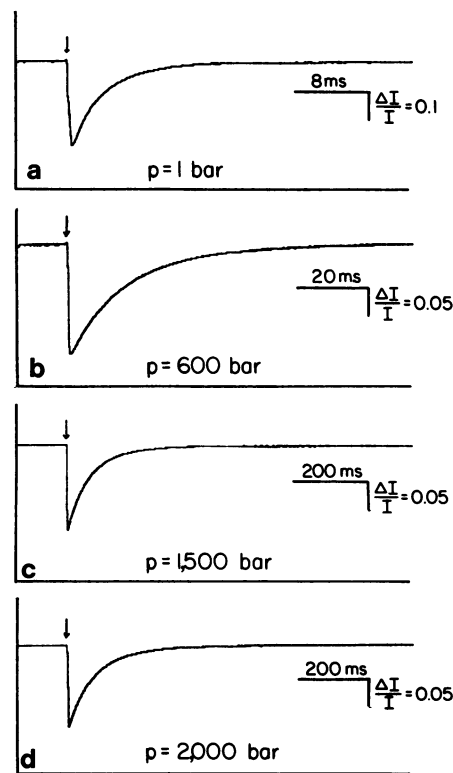

FIGURE 1 Flash-induced transmittance changes at 410 nm for PM sheets in 10 mM imidazole-HCl buffer, pH = 7.2, 25°C, at different pressures. a, 1 bar; b, 600 bar; c, 1,500 bar; d, 2,000 bar.
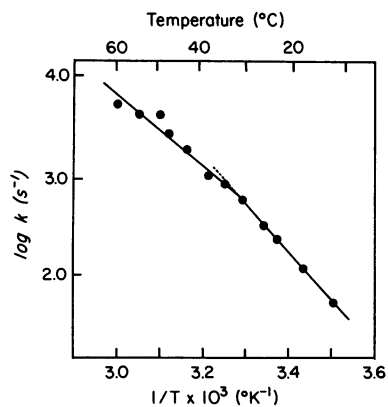
FIGURE 2 Arrehenius plot of the rate constant for the decay of the M intermediate in PM sheets, monitored at 410 nm, from data as in Fig. 1 *a*. 10 mM imidazole-HCl buffer, pH = 7.2, 25°C.

TABLE I
ACTIVATED VOLUME CHANGES ($\Delta V\ddagger$) FOR THE DECAY OF THE M PHOTOCYCLE INTERMEDIATE OF BACTERIORHODOPSIN AT DIFFERENT TEMPERATURES

Temperature	Lower-pressure phase	Higher-pressure phase
°C	ml/mol	ml/mol
3.5	55	7.3
12	57	7.0
23	57	7.4
31	53	7.5
41	48	7.7
50	43	13
60	39	16

of decay of M becomes smaller with increasing pressure up to ~1,000 bar (cf., Fig. 1 *a* and *b*). Above this pressure, it appears to be insensitive to pressure (see Fig. 1 *c* and *d*). It should be noted that kinetic analysis of M decay reveals two components, and both components have very similiar pressure dependencies (Tsuda, Ohno, Govindjee, Ebrey, in preparation). For simplicity, the rate constants used in this paper are just the reciprocal of the time for M to decay to 1/2 its initial amplitude. A plot of the logarithm of the rate constant against pressure for several temperatures is shown in Fig. 3. The plot is biphasic with a clear break. $\Delta V\ddagger$ is large at lower pressures and decreases above the break. Table I shows that $\Delta V\ddagger$ changes with temperature at pressures below the break, but there is no abrupt change near 30°C. On the other hand, the critical pressure, the pressure at which a sharp break in the relationship between the logarithm of the rate of the M decay and pressure is

observed, increased with increasing temperature (Fig. 3). A plot of the critical pressure (P_c) against temperature is shown in Fig. 4. The slope $dT/dP = 53$ $K/1,000$ atm and extrapolation to 1 atm gives -42°C. We suggest that this is the critical temperature of a lipid phase transition of the PM (see below and Discussion).

BR/DMPC Vesicles

To see if the extrapolation to 1 atm of the plot of the critical pressure vs. temperature did give the phase-transition temperature of the lipid matrix of BR, we studied the effect of temperature and pressure on the M decay rate in BR containing vesicles reconstituted with DMPC. The phase-transition temperature of the liquid-crystal transition of both pure (no BR) DMPC vesicles and BR/DMPC vesicles, similar to those we have used, have already been studied (18, 22, 23–25). The former has a transition at ~23.5°C while an Arrhenius plot of M decay for the latter shows apparent breaks at 21° and 12°C (Fig. 5).

The effect of pressure on the rate of M decay in BR/DMPC vesicles was studied at different temperatures. The plots in Fig. 6 are biphasic at high (47.8°C) and low
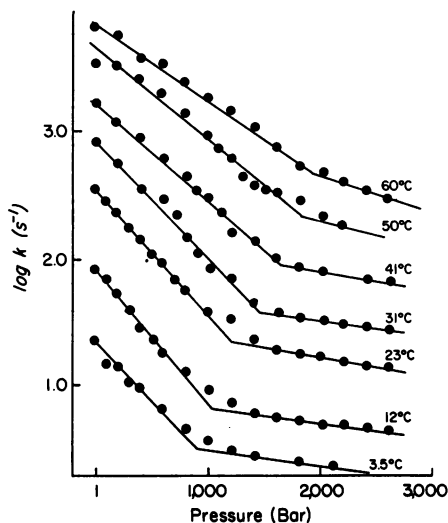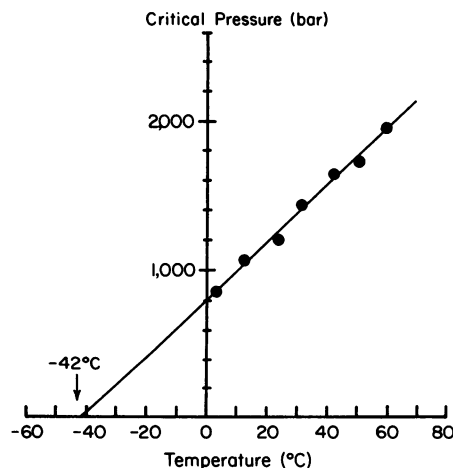

FIGURE 3 The effect of pressure on the rate constant of the M intermediate in PM sheets at different temperatures. 10 mM imidazole-HCl buffer, pH = 7.2.

FIGURE 4 Temperature dependence of the critical pressure as obtained-from plots in Fig. 3. 10 mM imidazole-HCl buffer, pH = 7.2.

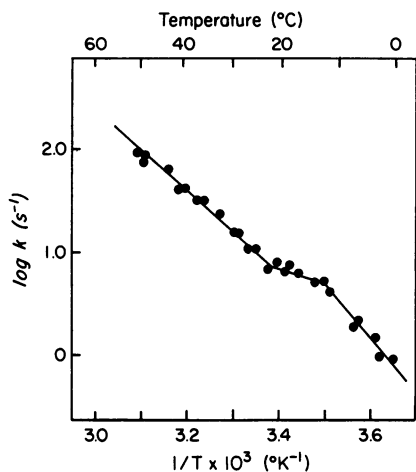TSUDA ET AL. *Phase Transition of the Purple Membrane*

FIGURE 5 Arrhenius plot of the rate constant of the M intermediate in BR/DMPC vesicles. 10 mM imidazole-HCl buffer, 150 mM KCl, pH = 7.2.

(12°C) temperatures and triphasic between these temperatures. There is a sharp break in the $\Delta V\ddagger$ at atmospheric pressure with $\Delta V\ddagger \simeq 0$ above 20.6°C and $\simeq 42$ ml below this temperature. In Fig. 7, the two breaks in the plots of the rate of M decay vs. pressure are plotted against temperature. The slopes dT/dP for the high-pressure and low-pressure regions are 24 $K/1,000$ atm and 19 $K/1,000$ atm, respectively. Extrapolation of the critical pressure to 1 atm in the lower-pressure region gives 18°C, which corresponds fairly well to the higher-temperature break point (21°C) in the Arrhenius plot of Fig. 5. The extrapolation of the critical-pressure value to 1 atm for the higher-pressure region gives −13°C, which does not correspond to either of the break points in the Arrhenius plot of Fig. 5 or to the phase-transition temperature of pure DMPC vesicles. This may be the phase-transition temperature of the mixed endogenous lipids/DMPC.

For comparison, the effect of pressure on the phase-

FIGURE 7 Temperature dependence of the critical pressure as obtained from plots in Fig. 6. (●), lower pressure phase; (o), higher pressure phase.

transition temperature of DMPC and BR/DMPC vesicles was studied by turbidity measurements. At 1 atm there is a phase transition at 23°C, close to what was measured above. For both DMPC vesicles and BR/DMPC vesicles, when the pressure increases, the transition temperature of the turbidity change increases. A plot of the transition temperature vs. pressure for both types of vesicles (Fig. 8) shows that their slopes are almost the same: $dT/dP = 20$ $K/1,000$ atm. Though the transition temperature T_c for the low pressure region in Fig. 7 (18°C) is slightly lower than that determined by the turbidity measurements (23°C), the slopes of the two plots are similar.

If, instead of the double-reconstitution procedure noted in the Materials and Methods section, the DMPC vesicles were only reconstituted a single time, they did not show any abrupt change in turbidity in the temperature range of 5°–50°C. They also gave just one break in the relationship between log k for M decay vs. pressure over this temperature range. Extrapolation of the critical pressure to 1 atm gives −30°C, far away from the T_c of pure DMPC vesicles and the T_c for doubly reconstituted vesicles. The slope
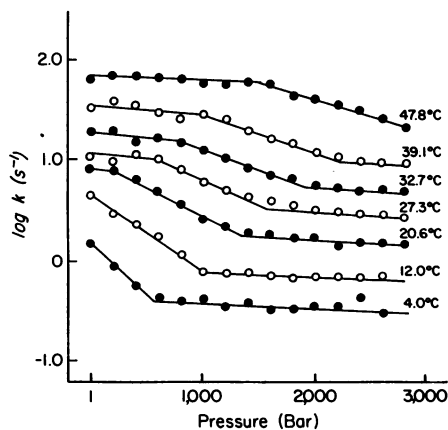

FIGURE 6 The effect of pressure on the rate constant of M decay in BR/DMPC vesicles at different temperatures. 10 mM imidazole-HCl buffer, 150 mM KCl, pH = 7.2.
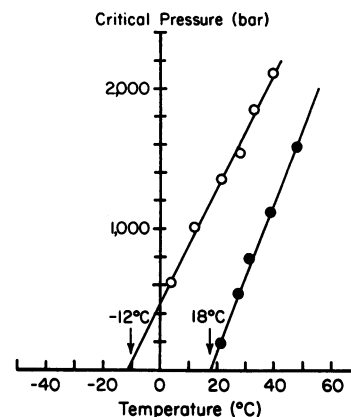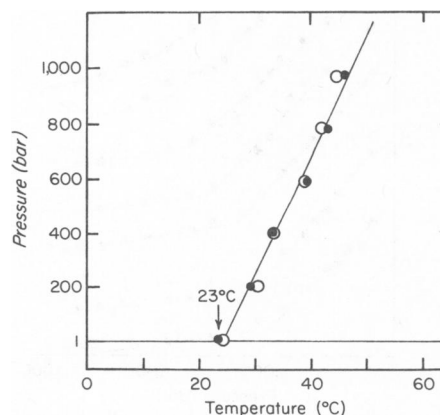

FIGURE 8 Pressure dependence of the transitions temperature of the turidity change measured at 460 nm in DMPC vesicles (o) and BR/DMPC (●) vesicles.

$dT/dP = 41\ K/1{,}000$ atm. These data suggest that the single reconstitution contains more native lipid of purple membrane than the double reconstitution.

In summary, a phase transition in BR/DMPC vesicles is found at ~20°C by two different methods: we also suggest that there may be a second transition at -13°C in our doubly reconstituted vesicles. The above results suggest that in BR/DMPC vesicles, the break in the plot of rate of M vs. pressure is due to a phase transition of the lipid, and the pressure vs. temperature at which this break takes place can be extrapolated to 1 atm, to give the phase-transition temperature at this pressure. Thus, when the temperature of the break in the half time of M decay vs. pressure for native PM is extrapolated to 1 atm, the transition temperature of about -42°C represents the temperature of a lipid phase transition for the native membrane.

DISCUSSION

The occurrence of an abrupt change in the slope of Arrhenius plot for a number of different phenomena involving membrane proteins has often been noted and correlated with phase transitions in the lipid surrounding the protein (14–16). Because the phase-transition temperature of lipids increases with increasing pressure, it is expected that the temperature at which a discontinuity in an Arrhenius plot occurs will increase with increasing pressure if the discontinuity is associated with a lipid phase transition.

In the case of Ca^{2+}-ATPase from sarcoplasmic reticulum (15, 16), extrapolation of the critical pressure to 1 atm gives the transition temperature that corresponds to a break point in the Arrhenius plot. Similar effects of pressure on the Arrhenius plot have been observed for other systems where phospholipids have been shown to be involved (15).

To see if such higher pressure methods can reveal a phase transition in purple membrane, we studied the pressure dependence of M decay at several temperatures in both native PM and PM reconstituted in DMPC, a lipid with a well-characterized transition temperature. A discontinuity in the Arrhenius plot of the M decay kinetics in BR/DMPC vesicles is found at ~ 20°C, which is close to the phase-transition temperature of pure DMPC vesicles. This thermotropic transition might be due to several things besides a phase transition. However, the activation volume ($\Delta V\ddagger$) near 1 atm for the M decay in the BR/DMPC vesicles has different values above and below the transition temperature, that is, $\Delta V\ddagger$ ~ 0 above 20.6°C and 42 ml below this temperature. Moreover, extrapolation of the critical pressure to 1 atm, for the low-pressure region, gives 18°C, which is close to the phase-transition temperature for DMPC vesicles. A similar transition temperature, 23°C, was obtained from the pressure dependence of the phase transition measured with light scattering. Thus, a variety of techniques, including the shift of the critical temperature with pressure, suggest that the discontinuity in the rate/pressure plots that shift with pressure represent a phase transition of BR/DMPC vesicles at ~20°C and 1 atm. This is close to the two transition temperatures at 17.5° and 23.5°C for BR/DMPC vesicles found by Heyn et al. (24) using differential scanning calorimetry.

A second break in the log k of M decay vs. pressure plot (Fig. 6) in doubly reconstituted BR/DMPC vesicles is found in the higher-pressure region. Above the critical pressure, $\Delta V\ddagger$ decreases and has a value of almost zero. Extrapolation of the critical pressure to 1 atm gives a temperature of -13°C, which is far below the phase-transition temperature of DMPC vesicles. The slope dT/dP is 24 $K/1{,}000$ atm. If the reconstitution was done just a single time, no low-pressure phase transition was observed from 5° to 50°C. The single high-pressure break in the M decay rate, when extrapolated to atmospheric pressure, gave a value of -30°C, shifted from the higher pressure DMPC value (-13°C) toward that of native purple membrane (-42°C). Thus, we propose that the extrapolated break at -13°C seen in doubly reconstituted vesicles is due to a mixed DMPC/native PM lipid phase transition.

With consideration to the above results, we analyzed the results of temperature and pressure effect on native PM sheets. As shown by other groups (6, 7), we also found a minor break in an Arrhenius plot of the M decay around 30°C (Fig. 2). However, we could not find any abrupt change in $\Delta V\ddagger$ below and above 30°C (Table I), suggesting this change is not due to a phase transition of the lipid. This break at 30°C may be a rather minor change in the rearrangement of the trimers of BR in the purple membrane such as Nakabayashi and Mihashi (26) have pointed out. The break in the relationship between pressure and log k of the M decay in PM sheets suggests that a pressure-induced phase transition does occur above 1,000 bar at around room temperature. Extrapolation of the critical pressure to 1 atm gives a temperature of -42°C. We suggest this is the phase-transition temperature of the native lipid of PM.

There exists no conclusive interpretation of the observed $\Delta V\ddagger$ in the reaction of any protein in solution. Model studies suggest that volume changes associated with solvation make significant contributions to the total $\Delta V\ddagger$ and should dominate over the other sources in most cases. Because $\Delta V\ddagger$ has a different value below and above the phase-transition temperature of the lipid, we suggest that solvation of BR with a lipid is a major contributor to the activated volume change of the M decay.

Finally, the large value (53 $K/1{,}000$ atm) of the slope dT/dP is notable. This is much higher than the slope (20–25 $K/1{,}000$ atm) usually seen for the phase transition of normal lipids (see Table IV in reference 15 and the present results for the higher-temperature DMPC vesicle transitions). This large slope may either be due to the very unusual lipids of the PM, the small lipid/protein ratio, or some influence of protein-protein interaction in the PM.

REFERENCES

1. Stoeckenius, W., R. H. Lozier, R. A. Bogomolni, 1979. Bacteriorhodopsin and the purple membrane of *Halobacteria. Biochim. Biophys. Acta.* 505:215–278.
2. Jost, P. C., D. A. McMillen, W. D. Morgan, and W. Stoeckenius. 1978. Lipid-protein interactions in the purple membrane. *In* Light Transducing Membranes. D. W. Deamer, editor. Academic Press, Inc., New York. 141–155.
3. Chignell, C. F., and D. A. Chignell. 1975. A spin label study of purple membranes from *Halobacterium halobium. Biochem. Biophys. Res. Commun.* 62:136–143.
4. Degani, H., D. Bach, A. Danon, H. Garty, M. Eisenbach, and S. R. Caplan. 1978. Phase transition of the lipids of *Halobacterium halobium. In* Energetics and Structure of Halophilic Microorganisms. S. R. Caplan, and M. Ginzburg, editors. Elsevier North-Holland, Inc., NewYork. 225–232.
5. Degani, H., A. Danon, and S. R. Caplan. 1980. Proton and carbon-13 nuclear magnetic resonance studies of the polar lipids of *Halobacterium halobium. Biochemistry.* 19:1626–1631.
6. Korenstein, R., W. V. Sherman, and S. R. Caplan. 1976. Kinetic isotope effects in the photochemical cycle of bacteriorhodopsin. *Biophys. Struct. Mech.* 2:267–276.
7. Hwang, S.-B., Y.-W. Tseng, and W. Stoeckenius. 1981. Spontaneous aggregation of bacteriorhodopsin in brown membrane. *Photochem. Photobiol.* 33:419–428.
8. Eisenbach, M., and R. Caplan. 1979. The light-driven proton pump of *Halobacterium halobium*: mechanism and function. *Curr. Top. Membr. Trans.* 12:165–248.
9. Jackson, M. B., and J. M. Sturtevant. 1978. The phase transitions of the purple membranes of *Halobacterium halobium. Biochemistry.* 17:911–915.
10. Hiraki, K., T. Hamanaka, T. Mitsui, and Y. Kito. 1981. Phase transitions of the purple membrane and the brown holomembrane. X-ray diffraction, circular dichroism, spectrum, and absorption spectrum studies. *Biochim. Biophys. Acta.* 647:18–28.
11. Bayley, W. T., and R. A. Morton. 1978. Recent developments in the molecular biology of extremely halophilic bacteria. *CRC Crit. Rev. Microbiol.* 6:151–505.
12. Lindsey, H., N. O. Peterson, and S. I. Chan. 1979. Physiochemical characterization of 1,2-biphytanoyl-*sn*-glycero-3-phosphocholine

in model membrane systems. *Biochim. Biophys. Acta.* 555:147–167.
13. Chen, J. S., P. G. Barton, D. Brown, and M. Kates, 1974. Osmotic and microscopic studies on bilayers of polar lipids from the extreme halophile, *Halobacterium cutirubrum. Biochim. Biophys. Acta.* 352:202–217.
14. Srinivasan, K. R., R. L. Kay, and J. F. Nagle. 1974. The pressure dependence of the lipid bilayer phase transition. *Biochemistry.* 13:3496–3496.
15. Heremans, K. 1982. High pressure effects on proteins and otherbiomolecules. *Annu. Rev. Biophys. Bioeng.* 11:1–21.
16. Heremans, K., and F. Wuytack. 1980. Pressure effect on the Arrhenius discontinuity in Ca^{++}-ATPase from sarcoplasmic reticulum: evidence for lipid involement. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 117:161–163.
17. Becher, B., and J. Y. Cassim. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium. Prep. Biochem.* 5:161–178.
18. Bakker, E. P., and S. R. Caplan. 1978. Phospholipid substitution of the purple membrane: the stoichiometry of light-induced proton release by phospholipid substituted purple membranes. *Biochim. Biophys. Acta.* 503:362–379.
19. Tsuda, M., and T. G. Ebrey. 1979. Effect of high pressure on the absorption spectrum and isomeric composition of bacteriorhodopsin. *Biophys. J.* 30:149–157.
20. Tsuda, M. 1982. Effect of pressure on visual pigment and purple membrane. *Methods Enzymol.* 88:714–722.
21. Tsuda, M., I. Shirotani, S. Minomura, and Y. Terayama. 1979. The effect of pressure on the dissociation of weak acids in aqueous buffers. *Bull. Chem. Soc. Jpn.* 49:2952–2955.
22. Tsuda, M., I. Shirotani, S. Minomura, and Y. Terayama. 1977. Pressure induced intermediates in the photochemical reaction of squid rhodopsin. *Biochem. Biophys. Res. Commun.* 76:989–999.
23. Cherry, R. J., U. Muller, R. Henderson, and M. P. Heyn. 1978. Temperature-dependent aggregation of bacteriorhodopsin in depalmitoyl- and demyristoylphosphatidylcholine vesicles. *J. Mol. Biol.* 121:283–298.
24. Heyn, M. P., R. J. Cherry, and N. A. Dencher. 1981. Lipid protein interactions in bacteriorhodopsin-dimyristoylphosphatidylcholine vesicles. *Biochemistry.* 20:840–849.
25. Heyn, M. P., A. Blume, M. Rehorek, and N. A. Dencher. 1981. Calorimetric and fluorescence depolarization studies on the lipid phase transition of bacteriorhodopsin-dimyristoylphosphatidylcholine vesicles. *Biochemistry.* 20:7109–7115.
26. Nakabayashi, M., and K. Mihashi. 1981. Fluorescence of 1-dimethylaminonaphthalene-5-sulfonate and pyrene-1-sulfonate conjugated to bacteriorhodopsin. *Photochem. Photobiol.* 33:444–454.