Effect of the Vesicular Stomatitis Virus Matrix Protein on the Lateral Organization of Lipid Bilayers Containing Phosphatidylglycerol: Use of Fluorescent Phospholipid Analogues†

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Received May 3, 1985

ABSTRACT: In order to investigate the mode of interaction of peripheral membrane proteins with the lipid bilayer, the basic (pI ~ 9.1) matrix (M) protein of vesicular stomatitis virus was reconstituted with small unilamellar vesicles (SUV) containing phospholipids with acidic head groups. The lateral organization of lipids in such reconstituted membranes was probed by fluorescent phospholipid analogues labeled with pyrene fatty acids. The excimer/monomer (E/M) fluorescence intensity ratios of the intrinsic pyrene phospholipid probes were measured at various temperatures in M protein reconstituted SUV composed of 50 mol % each of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG). The M protein showed relatively small effects on the E/M ratio either in the gel or in the liquid-crystalline phase. However, during the gel to liquid-crystalline phase transition, the M protein induced a large increase in the E/M ratio due to phase separation of lipids into a neutral DPPC-rich phase and DPPG domains presumably bound to M protein. Similar phase separation of bilayer lipids was also observed in the M protein reconstituted with mixed lipid vesicles containing one low-melting lipid component (1-palmitoyl-2-oleoylphosphatidylcholine or 1-palmitoyl-2-oleoylphosphatidylglycerol) or a low mole percent of cholesterol. The self-quenching of 4-nitro-2,1,3-benzoxadiazole (NBD) fluorescence, as a measure of lipid clustering in the bilayer, was also studied in M protein reconstituted DPPC–DPPG vesicles containing 5 mol % NBD-phosphatidylethanolamine (NBD-PE). The quenching of NBD-PE was enhanced at least 2-fold in M protein reconstituted vesicles at temperatures within or below the phase transition. All these experiments demonstrate that the binding of M protein in lipid bilayers containing acidic phospholipid head groups induces lateral reorganization of lipids in the membrane plane, an effect which is far more pronounced during the transition of membrane lipids from the liquid-crystalline to the gel state.

Vesicular stomatitis virus (VSV)[1] consists of a ribonucleocapsid core and a limiting membrane derived from the plasma membrane of infected host cells (Wagner, 1975). The envelope of VSV contains two proteins, an externally oriented integral glycoprotein (G protein) which serves as the cell recognition organ and the matrix (M) protein which lines the inner surface of the lipid bilayer in association with the nucleocapsid core (Schloemer & Wagner, 1975; Patzer et al., 1979; Zakowski & Wagner, 1980). The M protein appears to be involved in condensation of the viral nucleocapsid, presumably before budding of virus particles from the plasma membrane of infected cells (Newcomb et al., 1982).

Both G and M proteins of the viral membrane have been purified to homogeneity and reconstituted into phospholipid vesicles of defined lipid composition (Petri & Wagner, 1979; Zakowski et al., 1981). Recent studies performed in this laboratory and elsewhere have utilized the G protein reconstituted vesicles as a model system to study the interaction of a transmembrane protein with the lipid bilayer (Petri et al., 1980, 1981; Pal et al., 1983; Altstiel & Landsberger, 1981). On the other hand, reconstitution with vesicles of the peripheral M protein, which is highly basic (pI ~ 9.1; Carroll & Wagner, 1979), requires the presence of acidic phospholipids for its interaction with a lipid bilayer (Zakowski et al., 1981; Wiener et al., 1983a). Both steady-state and differential polarized phase fluorometric studies have demonstrated that the reconstitution of M protein with mixed phospholipid vesicles composed of 50 mol % dipalmitoylphosphatidylcholine (DPPC) and 50 mol % diplamitoylphosphatidylglycerol (DPPG) resulted in a dramatic increase in the lipid phase transition temperature and an apparent increase in the order of the lipids in the gel state (Wiener et al., 1983a). Further examination of the thermotropic behavior of DPPC–DPPG-fused unilamellar vesicles reconstituted with M protein by differential scanning calorimetry also revealed a profound effect of M protein on the lipid phase transition (Wiener et al., 1983b).

In this paper, we describe the effect of the matrix protein of VSV on the lateral organization of membrane lipids using

† This work was supported by Grants AI-21652, AI-11112, and HL-17576 from the National Institutes of Health, by Grant MV-9I from the American Cancer Society, and by Grant 2669 from U.S.–Israel Binational Science Foundation.

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[1] Abbreviations: VSV, vesicular stomatitis virus; G protein, glycoprotein; M protein, matrix protein; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; SUV, small unilamellar vesicle(s); POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; NBD-PE, 4-nitro-2,1,3-benzoxadiazolylphosphatidylethanolamine; BHK-21, baby hamster kidney 21 cells; $PyC_{10}PC$, 1-palmitoyl-2-pyrenyldecanoylphosphatidylcholine; $PyC_{10}PG$, 1-palmitoyl-2-pyrenyldecanoylphosphatidylglycerol; $PyC_{16}PC$, 1-palmitoyl-2-pyrenylhexadecanoylphosphatidylcholine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-7651$01.50/0

pyrene phospholipids as probes. These probes are becoming increasingly useful to study the lateral phase separation phenomena in model and biological membranes due to their capability to form excited-state dimers (excimers) in a temperature- and concentration-dependent process (Galla & Hartmann, 1980; Galla & Luisetti, 1980). The results presented here indicate that the M protein affects the lateral distribution of lipids in the membrane plane especially during the fluid–gel transition and such an effect can be attributed primarily to the strong interaction of M protein with head groups of acidic phospholipids.

Experimental Procedures

Materials

Lipids and Fluorescent Probes. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC), 1,2-dipalmitoyl-3-*sn*-phosphatidylglycerol (DPPG), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylglycerol (POPG) were obtained from Avanti Biochemicals, Birmingham, AL. Cholesterol was obtained from Supelco, Inc., Bellefonte, PA. Phospholipid stocks were judged to be pure when analyzed by thin-layer chromatography in a chloroform/methanol/ammonium hydroxide (65/25/4) solvent system. All lipid stocks were maintained at −80 °C in spectral-grade chloroform and were routinely assayed for total phosphorus content by a modification of the method of Marinetti (1962). L-α-dipalmitoyl[1-^{14}C]phosphatidylcholine ([^{14}C]DPPC; 100 mCi/mmol) was obtained from New England Nuclear, Boston, MA. 1-Pyrenenonanoic acid, 1-pyrenedecanoic acid, 1-pyrenedodecanoic acid, and 1-pyrenehexadecanoic acid were obtained from Molecular Probes, Junction City, OR.

Cells and Viruses. Baby hamster kidney 21 (BHK-21) cells were grown at 37 °C in Dulbecco's modified Eagle's (DME) medium supplemented with 15% tryptose/phosphate broth, 10% calf serum, and antibiotics as described elsewhere (Barenholz et al., 1976). Plaque-purified VS virus of the Indiana serotype was used to infect confluent monolayers of BKH-21 cells at a multiplicity of 0.2 plaque-forming unit/cell. The viral proteins were labeled by inclusion in the infection medium of 5 μCi/mL ^{3}H-labeled amino acid mixture (New England Nuclear, Boston, MA). Following 17-h incubation at 31 °C, bullet-shaped virions were collected from the supernatant medium and purified by differential, rate zonal, and isopycnic centrifugation in sucrose and potassium tartrate gradients as described elsewhere (Barenholz et al., 1976). Purified virus was stored in phosphate-buffered saline (pH 7.4) at −70 °C at a concentration of 2–5 mg/mL.

Methods

Isolation of the VSV M Protein and Reconstitution into Lipid Vesicles. The M protein of VSV was purified by the method of Zakowski et al. (1981). In brief, purified VS virus at a protein concentration of 0.5 mg/mL was solubilized in 10 mM Tricine (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl, and 1.2 mM dithiothreitol at room temperature for 30 min. Following centrifugation to pellet the nucleocapsid material, the supernatant fluid containing the viral G and M proteins and viral lipids was chromatographed over a Whatman P77 phosphocellulose column (1 × 5 cm) equilibrated with 10 mM Tricine (pH 7.5) containing 0.125 M NaCl and 10% glycerol. Following thorough column washing, the M protein was eluted in equilibration buffer containing 0.65 M NaCl and stored at 4 °C. Purified M protein isolated in this manner was 98% pure as determined by SDS–polyacrylamide gel electrophoresis.

Purified viral M protein was reconstituted with preformed sonicated vesicles containing equimolar proportions of phosphatidylcholine and phosphatidylglycerol exactly as previously described (Wiener et al., 1983a). In brief, dried phospholipid mixtures containing the desired mole percent of fluorescent phospholipid analogue and trace quantities of [^{14}C]DPPC were lyophilized for 12 h in the dark and resuspended in 4 mL of 10 mM Tricine (pH 7.5) containing 0.55 M NaCl. Dissociation of the lipid mixture was performed by vortexing at a temperature of 5–7 °C above the lipid phase transition temperature (T_m) under nitrogen in the dark. Small unilamellar vesicles were made from this lipid suspension by sonication as described elsewhere (Wiener et al., 1983a). Viral M protein was added to the vesicles for the desired M protein/phospholipid molar ratio, and the mixture was dialyzed against 4 L of 10 mM Tricine (pH 7.5) in the dark under an N_2 atmosphere for 24 h at the desired temperature with two changes of buffer. Sucrose density gradient centrifugation of reconstituted vesicles showed that the binding of M protein was uniform with respect to the vesicle population and resulted in an increase in density proportional to the mole percent of M protein bound. The M protein binds solely to the outer leaflet of the vesicle bilayer under the conditions of this reconstitution protocol (Zakowski et al., 1981). Moreover, the association of M protein with the lipid bilayer was unaffected by temperature. Protein/phospholipid molar ratios were calculated by using a molecular weight for M protein of 29 000. protein concentrations were determined by the method of Lowry et al. (1951) and by the specific activity of ^{3}H-amino acid labeled M protein. Phospholipid concentration was determined as described by Marientti (1962).

Synthesis of Phospholipids Labeled with Pyrene Fatty Acids. (A) 1-Palmitoyl-2-pyrenyldecanoyl-3-sn-phosphatidylcholine ($PyC_{10}PC$). The synthesis of pyrene fatty acid labeled phosphatidylcholine followed the procedure of Mason et al. (1981) with a minor modification. In brief, pyrene fatty acid anhydride was prepared with the respective pyrene fatty acid by reaction with dicyclohexylcarbodiimide (Aldrich, Milwaukee, WI) as described by Selinger & Lapidot (1966). L-α-Palmitoyllysophosphatidylcholine (Sigma, St. Louis, MO) and the catalyst 4-pyrrolidinopyridine (Aldrich) were added in CCl_4, and the reaction mixture was incubated for 16 h at 37 °C in the dark with stirring. The pyrene-containing PC derivative was purified free of unreacted materials by thin-layer chromatography on silica gel plates in the solvent system $CHCl_3$/MeOH/NH_3 (62/25/4 v/v). Following elution from the silica gel with methanol/chloroform/H_2O (88/10/2 v/v), the probe was dried under N_2 and resuspended in spectral-grade chloroform, and the concentration was determined spectrophotometrically by using a molar extinction coefficient of 50 000 mol cm^{-1} at 347 nm (Pownall & Smith, 1973). The purified pyrene fatty acid labeled PC comigrated with a POPC standard in TLC. Digestion of purified probe with phospholipase A_2 liberated free pyrene fatty acid and nonfluorescent lysolecithin. Fluorescent PC derivatives were stored at −70 °C in the dark under a nitrogen atmosphere.

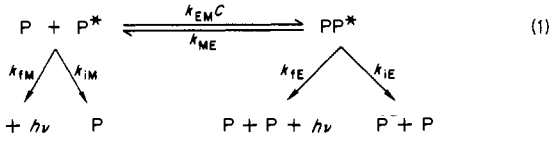
(B) 1-Palmitoyl-2-pyrenyldecanoyl-3-sn-phosphatidylglycerol ($PyC_{10}PG$) was synthesized from 1-palmitoyl-2-pyrenyldecanoyl-3-*sn*-phosphatidylcholine ($PyC_{10}PC$) by the method of Comfurius & Zwaal (1977). Briefly, 1 μmol of purified $PyC_{10}PC$ was dried under a stream of nitrogen, dissolved in 5.0 mL of diethyl ether (Sigma), and added to an equal volume of 50 mM sodium acetate buffer (pH 5.6) containing 50 mM $CaCl_2$, 50% glycerol, and 5 units of cabbage phospholipase D (0.3 unit/mg; Calbiochem-Behring, San

Diego, CA). The reaction mixture was stirred continuously at 37 °C in the dark in a distillation condenser to decrease the evaporation of ether. Additional enzyme and ether were added as needed. The reaction was terminated after 2 h by the addition of 10 mL of 100 mM EDTA and the ether evaporated under a stream of nitrogen. The aqueous portion of the reaction was extracted with chloroform/methanol (2/1 v/v) (Folch et al., 1957), and the chloroform phase was evaporated. The resulting material was subjected to thin-layer chromatography in the solvent system described above. The region of the plate containing pyrenyl-PG was identified by UV illumination and compared with an iodine-stained POPG standard comigrating in a parallel lane on the plate. Following elution of the probe from the silica gel, its concentration in chloroform was determined by using a molar extinction coefficient of 50 000 mol cm^{-1} at 347 nm (Pownall & Smith, 1973). Phospholipase A_2 treatment of an aliquot of the purified probe resulted in the liberation of free pyrenedecanoic fatty acid and nonfluorescent lysophosphatidylglycerol, suggesting that the pyrene fatty acid was present in the β-position. The fluorescent lipid was stored in chloroform at −70 °C in the dark under a nitrogen atmosphere and used within 10 days of synthesis.

Fluorescence Studies. Temperature-dependent fluorescence emission experiments were performed in a Perkin-Elmer MPF3 fluorescence spectrophotometer equipped with a Lauda thermoregulated bath circulator. The temperature of the sample was monitored continuously to within 0.1 °C by a thermistor attached a digital ohmmeter. Samples under a nitrogen atmosphere were heated to the desired temperature and the emission spectra monitored following equilibration to the lower temperature. Determination of the pyrene fluorescence emission intensity used in the calculation of excimer/monomer ratios was obtained by excitation of the vesicle preparations at 347 nm; the emission spectrum was monitored between 380 and 500 nm by using the MPF3 350-nm cutoff filter to reduce scattered light. The vesicle preparation containing the NBD-PE probe was excited at 475 nm, and fluorescence emission (F) was measured at 530 nm. The fluorescence emission in the absence of quenching (F_0) was determined at 530 nm in the presence of 0.8% Triton X-100 as described by Hoekstra (1982).

Results

Principles. Fluorescence spectroscopic properties of pyrene probes have been described in recent reviews (Galla & Hartmann, 1980; Lakowicz, 1981). A typical pyrene fluorescence emission spectrum is characterized by emission maxima around 385 and 470 nm for the excited-state monomer and excimer, respectively. The kinetic processes involved in the excimer formation were reviewed recently by Galla & Hartmann (1980) and can be summarized as



(1)

where P* and PP* are excited-state monomer and excimer molecules, respectively, k_{fE} and k_{fM} are the excimer and monomer fluorescence decay parameters, respectively, and k_{iM} and k_{iE} are the radiationless decay parameters for monomer and excimer species, respectively. $k_{EM}C$ is the rate constant of excimer formation, C is the probe concentration, and k_{ME} is the rate constant of regeneration of P* from the dissociation of PP*.

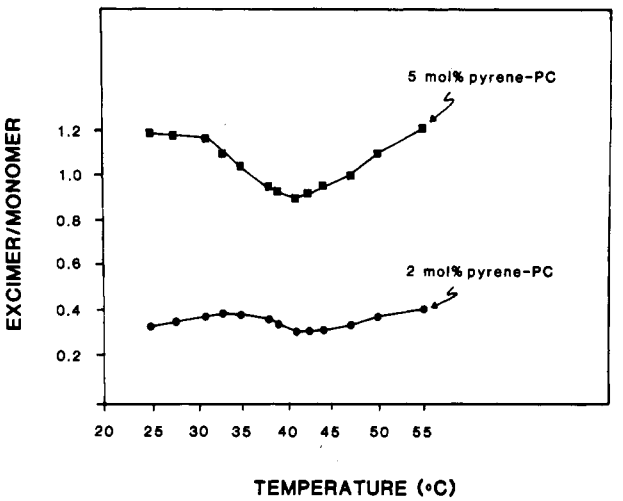


FIGURE 1: Excimer/monomer ratios studied by pyrenyl-PC fluorescence emission vs. temperature at two concentrations of pyrenyl-PC incorporated into DPPC SUV. Pyrenyl-PC ($PyC_{16}PC$) was incorporated into DPPC SUV by cosonication. The excimer and monomer peak heights were measured from the emission maxima at 470 and 385 nm, respectively. Excitation was performed at 347 nm. The measured intensities were corrected for the fluorescence emission of vesicles in the absence of probe. Vesicles were equilibrated at 55 °C and pyrene emission spectra taken after reequilibration to the lower temperature.

The E/M fluorescence intensity ratio often serves as a direct measure of the concentration-dependent collision rate of pyrene molecules and therefore has been utilized to investigate lateral phase separation in bilayers and lateral as well as transversal mobility of phospholipids in the membrane, and also in the study of lipid–protein interactions in both natural and model membranes (Hartmann & Galla, 1978; Galla et al., 1979; Galla & Hartmann, 1980; Galla & Luisetti, 1980; Dangreau et al., 1982; Freire et al., 1983; Roseman & Thompson, 1980). In an effort to characterize the effect of M protein on the lateral organization of lipids in the bilayer, the protein was reconstituted into mixed phospholipid vesicles containing pyrene phospholipid analogues. The effect of temperature on the E/M fluorescence intensity was measured in a variety of vesicle systems. It is assumed that the fluorescence process of pyrene phospholipid probes described in eq 1 is unaffected by the presence of M protein in the bilayer. The amino acid sequence of M protein has been deduced from cDNA clones that contained the complete coding sequence of the mRNA encoding the matrix protein (Rose & Gallione, 1981). The protein is highly basic in nature ($pI \simeq 9.2$) with no obvious hydrophobic stretches of amino acids in the molecule. Thus, it is very unlikely that a specific interaction of the hydrophobic pyrene probes with M protein took place during these fluorescence measurements.

Concentration and Miscibility of Pyrene Phospholipid Probes in Vesicles. Initially, it was necessary to determine the optimal condition of probe necessary for observable excimer formation. The pyrene C_{16} fatty acid linked PC ($PyC_{16}PC$) was incorporated into vesicles by cosonication with DPPC. Figure 1 illustrates the E/M ratio of $PyC_{16}PC$ at different temperatures in DPPC vesicles containing 2 and 5 mol % $PyC_{16}PC$. In vesicles containing 5 mol % $PyC_{16}PC$, the E/M ratio was 3–5-fold higher than that in vesicles containing 2 mol % probe. Both the curves exhibited a similar pattern with a constant E/M ratio in the gel state and a drop in the E/M ratio as the DPPC molecules started melting around 33 °C. This drop in the E/M ratio during the gel–fluid transition was more pronounced in the vesicles containing 5 mol % $PyC_{16}PC$.

Table I: Excimer/Monomer Ratios in Preformed Sonicated Vesicles Containing 2 mol % 1-Palmitoyl-2-pyrenylhexadecanoyl-3-*sn*-phosphatidylcholine Reconstituted in the Absence or Presence of M Protein

vesicle lipid compn (%)[a]		E/M ratios at different temp[b] (°C)								
		20			41			60		
DPPG	DPPC	no M	1/350 M	1/175 M	no M	1/350 M	1/175 M	no M	1/350 M	1/175 M
75	25	0.31	0.76	0.76	0.55	1.54	1.63	0.67	0.76	0.79
50	50	0.33	0.68	0.73	0.55	1.24	1.26	0.62	0.69	0.72
25	75	0.31	0.54	0.65	0.56	0.92	1.11	0.65	0.70	0.70

[a] Mole percent concentrations of DPPG and DPPC. [b] Determinations made at temperatures in the gel state (20 °C), at the phase transition (41 °C), and in the liquid-crystalline phase (60 °C).

Since the fluorescent phospholipid probes with their bulky pyrene moiety preferred to partition into the fluid domain of the membrane (B. Lentz, The University of North Carolina, personal communication), the local concentration of the fluorophore in various domains of the bilayer could not be obtained directly from the total content of fluorophore present in the membrane. In vesicles containing 5 mol % $PyC_{16}PC$, the large excess of pyrene probe could not be accommodated in the gel state, and the fluorophore presumably phase-separated to form its own local environment which was responsible for a 5-fold difference of the E/M ratio (expected to be 2.5-fold) in the gel state between the two vesicles containing 2 and 5 mol % $PyC_{16}PC$. This also explains the large drop in the E/M ratio observed during melting of DPPC molecules in vesicles containing 5 mol % $PyC_{16}PC$ as the change in the local concentration of the probe was higher as the fluorophore moved from its own domain into the DPPC melting phase. The increase in the E/M ratio above 40 °C in the fluid phase was mainly due to an increase in the diffusion of pyrene molecules with temperature. This experiment thus suggests that pyrene phospholipid probe at a level of 5 mol % in the bilayer behaves like a separate component which tends to form its domain especially in the gel state. To minimize the perturbation in the bilayer by pyrene molecules, subsequent experiments were carried out with a probe concentration of 2 mol % in the vesicles.

The miscibility of $PyC_{16}PC$ in DPPC–DPPG mixed vesicles was determined by incorporation of the pyrene probe at the level of 2 mol % in DPPC–DPPG small unilamellar vesicles (SUV) at different concentrations of the two phospholipid components. Table I presents the results of experiments in which the E/M ratios were determined in the gel state (20 °C), in the liquid-crystalline state (60 °C), and at the phase transition temperature (41 °C). In DPPC–DPPG vesicles, the E/M ratios were unaffected by the content of PC in the bilayer, suggesting that the PyPC was miscible in the membrane matrix with both lipid components.

DPPC–DPPG Vesicles Reconstituted with M Protein. DPPC–DPPG vesicles (1/1 molar ratio) containing 2 mol % $PyC_{10}PC$ or $PyC_{10}PG$ were reconstituted with VSV M protein, and the E/M ratio was measured as a function of temperature. It is to be noted that both phospholipids have identical melting temperature (T_m) (Silvius, 1982). As illustrated in Figure 2, both probes detected only minimal increases in the E/M ratio in the fluid state although PyPC detected a slightly larger increase in this ratio than did the PyPG probe. As excimerization of pyrene molecules in the fluid state of a bilayer is a diffusion-controlled process, this difference in the two probes is probably due to the fact that PyPG is bound to M protein and is therefore restricted in its mobility in the membrane plane as compared to free PyPC. During the phase transition, the E/M ratios increased markedly in vesicles containing M protein in systems probed by either PyPC or PyPG. This is attributable to the fact that with a decrease in temperature,

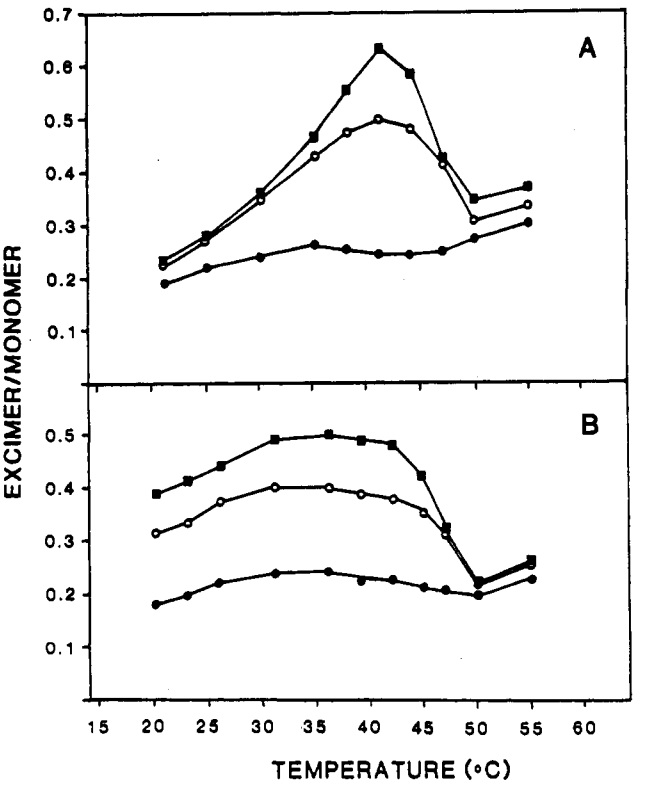


FIGURE 2: Pyrene phospholipid fluorescence emission calculated as excimer/monomer ratios in DPPG/DPPC (1/1) SUV in the presence and absence of the VS viral M protein. The fluorescence emission of 2 mol % pyrenyl-PC ($PyC_{10}PC$, panel A) or pyrenyl-PG ($PyC_{10}PG$, panel B) incorporated into DPPG/DPPC (1/1) SUV was measured with respect to temperature. Fluorescence measurements were performed as described under Experimental Procedures and Figure 1. (●) No M protein; (○) 0.125 mol % M protein; (■) 0.66 mol % M protein.

the rate of excimer formation (k_{EM}) decreased but the local concentration of probe (C) increased markedly due to phase separation of lipids into a neutral PC phase and an acidic PG phase to which M protein is bound. Under such conditions, the concentration term (C) dominates the $k_{EM}C$ product, resulting in an increase in the E/M ratio with a decrease in temperature. In the gel state, PyPC reported an E/M ratio which was only slightly higher than that observed in the absence of M protein. In contrast, in vesicles containing M protein, PyPG reported an elevated E/M ratio which remained higher well below the transition temperature. These results indicate that, in the gel state, PyPG occupied more packed PG-rich domains bound to M protein while the PyPC probe partitioned into bulk lipid.

The phase separation induced by M protein in DPPC–DPPG vesicles was further studied in lipid bilayers containing various concentrations of the acidic phospholipids, and the results are presented in Table I. The E/M ratios of $PyC_{16}PC$ in DPPC–DPPG vesicles were measured in the absence or in

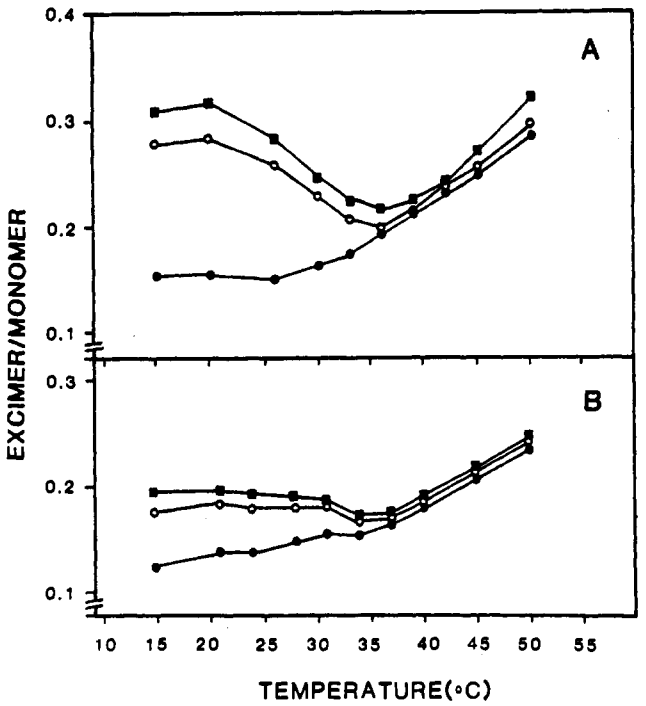


FIGURE 3: Pyrenyl-PC fluorescence emission calculated as excimer/monomer ratios in (A) DPPC/POPG (1/1) or (B) POPC/DPPG (1/1) SUV in the absence or presence of M protein. The fluorescence emission of 2 mol % pyrenyl-PC ($PyC_{10}PC$) incorporated into DPPC/POPG (1/1) SUV (panel A) or POPC/DPPG (1/1) SUV (panel B) was measured with respect to temperature. Fluorescence measurements were performed as described in Figure 1. (●) No M protein; (○) 0.33 mol % M protein; (■) 1.0 mol % M protein.

the presence of M protein in the gel state (20 °C) in the liquid-crystalline state (60 °C), and during the fluid–gel transition (41 °C). In the absence of M protein, PyPC was found to be miscible with both lipid components at all temperatures. The presence of M protein at levels of 0.28 and 0.57 mol % did not affect the miscibility of the probe to a large extent in the gel or in the fluid phase. In sharp contrast, during the fluid–gel transition, PyPC was found to be phase-separated into a PC-rich domain as evident from the increase in the E/M ratio from 1.11 to 1.63 in vesicles containing 0.57 mol % M protein when the PC content in the bilayer was decreased from 75 to 25 mol %. Similar effects were observed when $PyC_{10}PC$ was used as a probe in place of $PyC_{16}PC$ (data not shown). All these experiments suggest that in DPPC–DPPG mixed vesicles, the M protein induces lateral phase separation in the lipid bilayer into a DPPC bulk phase and M protein bound DPPG phase and that this effect was more pronounced during the fluid–gel transition.

POPC–DPPG and DPPC–POPG Vesicles Reconstituted with M Protein. These experiments were designed to test the hypothesis that M protein induced lateral redistribution of bilayer lipids would occur in mixed vesicles containing one low-temperature-melting lipid component and that a pyrenyl-PC probe would monitor the phase transition of membrane regions enriched in the neutral PC component. To this end, M protein was reconstituted into small unilamellar vesicles containing DPPC–POPG or POPC–DPPG, and the E/M ratio was measured as a function of temperature. As presented in Figure 3A, the $PyC_{10}PC$ probe reported a large increase in the E/M ratio in the gel state in DPPC–POPG vesicles reconstituted with M protein. The increase in the E/M ratio in the gel state was less pronounced in POPC–DPPG vesicles containing M protein (Figure 3B). In the liquid-crystalline state, no detectable difference in the E/M ratio was observed

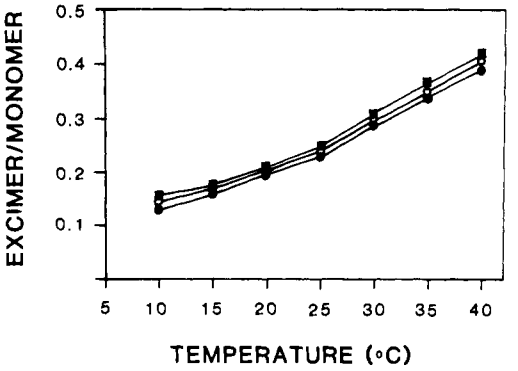


FIGURE 4: Pyrenyl-PC fluorescence emission (excimer/monomer ratios) in POPC/POPG (1/1) SUV in the presence or absence of the VS viral M protein. The fluorescence emission of 2 mol % $PyC_{10}PC$ incorporated into POPG/POPC (1/1) was measured in SUV with respect to temperature. Fluorescence measurements were performed as described in Figure 1. (●) No M proteins; (○) 0.125 mol % M protein; (■) 0.66 mol % M protein.

in either POPC–DPPG or DPPC–POPG vesicles in the absence or presence of M protein. In POPC–DPPG vesicles, the $PyC_{10}PC$ partitioned into the fluid POPC phase during the phase transition and the melting of the M protein bound DPPG phase had relatively little effect on the E/M ratio. In DPPC–POPG vesicles, the PyPC probe could partition either into the DPPC phase or into the more fluid M protein bound POPG phase. As the pyrene molecules prefers to be in the fluid region of the membrane (B. Lentz, The University of North Carolina, personal communication), quite likely it was present in the POPG phase. The E/M ratios of PyPC in these vesicles in the presence of M protein were slightly higher in DPPC–POPG vesicles than they were in the POPC–DPPG system, especially in the gel state. This may be attributed to the fact that PyPC was phase-separated into either the DPPC gel state or the M-bound POPG phase which had less volume than the POPC fluid phase in POPC–DPPG vesicles. A greater concentration of $PyC_{10}PC$ in such a compact volume presumably increased the $k_{EM}C$ product, thus resulting in a higher E/M ratio. During the phase transition, the DPPC lipids in DPPC–POPG vesicles melted with an increase in temperature, which would lower the local concentration of the probe, thus reducing $k_{EM}C$ and decreasing the E/M ratio significantly. In the liquid-crystalline state, the temperature-dependent increase in diffusion would dominate the k_{EM} product, resulting in an increase in the E/M ratio. These results again suggest that binding of M protein to lipid bilayers results in the redistribution of lipids into PG-rich domains bound to M protein and a PC-rich bulk lipid phase. Such an effect manifests itself during the fluid–gel transition when the $PyC_{10}PC$ probe tends to partition into a region of the bilayer which is less rigid during phase separation.

M Protein Reconstituted with POPC–POPG Vesicles. It was of interest to determine more precisely whether the interaction of M protein with PC–PG vesicles also induces lateral phase separation in the liquid-crystalline state. To this end, M protein was reconstituted with POPC–POPG vesicles containing $PyC_{10}PC$, and the E/M ratios were determined as a function of temperature (Figure 4). The E/M ratio increased at higher temperatures due to an increase in diffusion of the pyrene probe in vesicles with or without M protein. The M protein had no effect on the E/M ratio throughout the temperature range studied. These experiments thus indicate that the M protein induced lateral phase separation occurs only during the fluid–gel phase transition of membrane lipids and no detectable reorganization of lipids could be observed in the

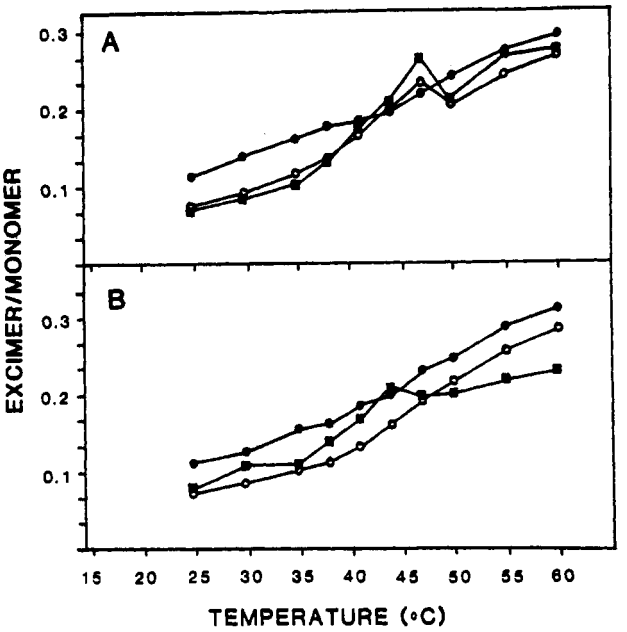


FIGURE 5: Pyrenyl-PC fluorescence emission (excimer/monomer ratios) in DPPG/DPPC (1/1) SUV containing cholesterol. The fluorescence emission of 2 mol % pyrenyl-PC ($PyC_{10}PC$) incorporated into DPPG/DPPC (1/1) SUV containing 5% cholesterol (panel A) or 15% cholesterol (panel B). Fluorescence measurements were performed as described in Figure 1. (●) No M protein; (○) 0.25 mol % M protein; (■) 0.5 mol % M protein.

fluid state. The M protein bound to the PG domain apparently is miscible in the bulk PC phase in the liquid-crystalline state.

M Protein Reconstituted with DPPC–DPPG Vesicles Containing Cholesterol. Cholesterol is an important component of the VSV membrane, representing nearly 35 mol % of total viral lipids (Patzer et al., 1979). Data from this laboratory have shown that cholesterol is distributed asymmetrically between the two layers of the viral membrane (Patzer et al., 1978; Pal et al., 1981). It was of interest to determine if the lateral redistribution of membrane phospholipids induced by M protein could occur in the presence of cholesterol which is known to affect the order of the bilayer lipids markedly both in the gel and in the liquid-crystalline state (DeKruijff et al., 1972). To this end, M protein was reconstituted into DPPC–DPPG vesicles containing 5 or 15 mol % cholesterol, and the E/M ratio of intrinsic $PyC_{10}PC$ probes was measured at various temperatures. As illustrated in Figure 5, cholesterol reduced the M protein induced lateral phase separation in the bilayer, and such an effect was proportional to the concentration of cholesterol present in the membrane. As shown in Figure 5A, vesicles containing 5 mol % cholesterol and reconstituted with 0.25 or 0.5 mol % M protein exhibited a small but significant increase in the E/M ratio at 47 °C during the fluid–gel transition. The E/M ratio was decreased in M protein reconstituted vesicles both above and below the phase transition temperature when compared with vesicles in the absence of M protein. However, in the presence of 15 mol % cholesterol, the effect of M protein on the E/M ratio of $PyC_{10}PC$ was significantly less than that observed in the presence of 5 mol % cholesterol (Figure 5B). This effect of cholesterol on the protein-induced lateral phase separation can be attributed to the fact that in the presence of cholesterol the phase transition of lipids in the bilayer is markedly diminished (Estep et al., 1978). As M protein induced phase separation in the membrane was manifested mainly during the fluid–gel transition, this effect was markedly diminished in the presence of cholesterol in the bilayer.

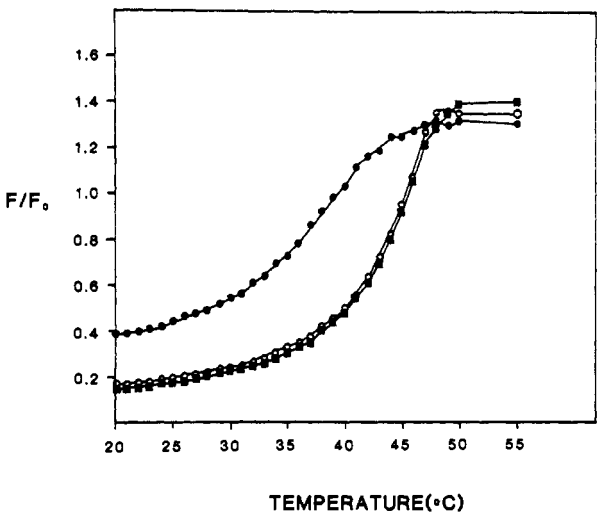


FIGURE 6: NBD-PE fluorescence quenching (F/F_0) in DPPG/DPPC (1/1) SUV in the absence or presence of M protein. NBD-PE was incorporated into SUV by consonication with vesicle lipids. Vesicles were equilibrated at 55 °C, and the fluorescence emission (F) at 530 nm was monitored at decreasing temperatures after excitation at 475 nm. The fluorescence emission in the absence of quenching (F_0) was determined at 530 nm in the presence of 0.8% Triton X-100, as described by Hoekstra (1982). (●) No M protein; (○) 0.1 mol % M protein; (■) 1.0 mol % M protein.

M Protein Reconstituted with DPPC–DPPG Vesicles Containing 4-Nitro-2,1,3-benzoxadiazolylphosphatidylethanolamine (NBD-PE). Phospholipid analogues containing the probe 4-nitro-2,1,3-benzoxadiazole (NBD) attached to either head groups or acyl chains have been used to study Ca^{2+}-induced phase separation in phosphatidylserine-containing lipid vesicles (Hoekstra, 1982). This method is based on the fact that during phase separation of membrane lipids, these NBD phospholipids concentrate in restricted domains in the bilayer. This increase in the local concentration of the probe in the membrane results in self-quenching. The NBD phospholipid analogue used here was NBD-PE in which the NBD group was covalently attached to the head group of the phospholipid. Small unilamellar vesicles of DPPC–DPPG containing 5 mol % NBD-PE were prepared by cosonication of the probe with the lipids, which were then reconstituted with the M protein. Figure 6 illustrates the degree of quenching (F/F_0) of NBD-PE induced in vesicles by M protein as a function of temperature. In the absence of M protein, the probe reported an increase in fluorescence quenching with decrease in temperature throughout the phase transition. In the presence of 0.1 or 1 mol % M protein in the vesicles, the degree of such quenching was enhanced nearly 2–3-fold mainly during the fluid–gel transition. However, no such effect was observed in the liquid-crystalline state in vesicles containing M protein. Although it was not possible to determine the mode of partitioning of NBD-PE in the lipid bilayer containing DPPC and DPPG, the vesicles studied here showed clearly that quenching of NBD-PE was enhanced markedly in bilayers containing M protein, presumably due to phase separation of membrane lipid, which resulted in an increase in the local concentration of the probe in the restricted domains of the bilayer. Since the highly basic matrix protein has no significant stretches of hydrophobic amino acids in the molecule (Rose & Gallione, 1981), it is quite unlikely that there is any specific interaction of M protein with hydrophobic NBD probes. Indeed, similar self-quenching of NBD-PE was observed in M protein reconstituted DPPC–DPPG vesicles containing 5 mol % cholesterol, as well as in POPC–DPPG

vesicles and in DPPC–POPG vesicles (data not shown).

Discussion

Earlier investigations from this laboratory using steady-state and differential polarized fluorometric studies have demonstrated that reconstitution of the basic M protein of VSV with DPPC–DPPG mixed vesicles resulted in a dramatic increase in the lipid phase transition temperature (T_m) as well as an apparent increase in the order of the bilayer in the gel state (Wiener et al., 1983a). Subsequent analysis by scanning calorimetry of the thermotropic behavior of DPPC–DPPG fused unilamellar vesicles has shown a profound effect of M protein on the fluid–gel transition temperature even at very low protein to lipid molar ratios. At relatively higher concentrations of M protein in the vesicles, the heat capacity function in calorimetric scans showed the presence of two superimposed peaks, presumably representing the melting of free and bound lipid species (Wiener et al., 1983b). These preliminary observations on the phase separation induced by M protein were examined in detail in this paper using excimer-forming pyrene phospholipid analogue and NBD-PE.

The experiments presented here demonstrate quite clearly that the interaction of M protein with a mixture of PC and PG vesicles resulted in lateral phase separation of individual lipid species into stable clustered domains. Using a variety of vesicle systems, it has been demonstrated here that the lateral reorganization of bilayer lipids induced by the M protein was observed mainly during a fluid–gel phase transition and this effect was diminished in the presence of cholesterol. Although the bulky pyrene-containing phospholipid molecules tend to concentrate in the fluid region of the bilayer, its distribution in the membrane as described in the present study was found to depend upon the type of the pyrene phospholipid probes used. Thus, when PyPC was used as a probe, it was concentrated in the neutral PC phase in M protein induced phase-separated lipid bilayer. On the other hand, a PyPG probe with its negatively charged head group apparently partitioned into the M protein bound PG phase which was more rigid than the bulk PC phase. Although the clustering of NBD-PE was increased markedly in the M protein reconstituted lipid vesicles, its mode of distribution into the phase-separated domains is not as clearly understood. These experiments thus demonstrate that while the fluorescent phospholipid analogues are useful probes to study the phase separation in the membrane matrix induced by a peripheral protein, the pattern of distribution of the probes into various domains depends upon the type of lipids and protein used for such study.

Ion- and protein-induced lateral phase separations in mixed phospholipid bilayers containing acidic phospholipids have also been demonstrated in a few other systems (Verkleij et al., 1974; Jacobson & Papahadjopoulos, 1975; Birrell & Griffin, 1976; Boggs et al., 1977; Hartmann & Galla, 1978; Galla & Hartmann, 1980). Birrell & Griffith (1976) have postulated that cytochrome *c* induced phase separation in diphosphatidylglycerol-containing bilayers may represent a method of controlling the rate of electron transport in the diphosphatidylglycerol-rich mitochondrial membrane. Lateral phase separation was also observed in LM cell plasma membranes near the cell growth temperature (Rintoul et al., 1979). The highly asymmetric distribution of PS in the inner leaflet of the VSV membrane and its possible interaction with M protein may be involved in lateral redistribution of bilayer lipids in viral membranes (Patzer et al., 1978). Further, during budding of virus particles from the plasma membrane of infected host cells, the M protein may be involved in phase separation of bilayer lipids which may play an important role in virus assembly.

Registry No. DPPG, 7091-44-3; DPPC, 63-89-8; POPG, 62600-81-1; POPC, 26853-31-6; $PyC_{10}PC$, 95864-17-8; PyC_{10} acid anhydride, 98874-80-7; L-α-palmitoyllysophosphatidylcholine, 17364-16-8; cholesterol, 57-88-5.

References

Altstiel, L. D., & Landsberger, F. R. (1981) *Virology 115*, 1–9.

Barenholz, Y., Moore, N. F., & Wagner, R. R. (1976) *Biochemistry 15*, 3563–3570.

Birrell, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925–2929.

Boggs, J. M., Moscarello, M. A., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 5420–5426.

Carroll, A. R., & Wagner, R. R. (1979) *J. Virol. 29*, 134–142.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Dangreau, H., Joniau, M., DeCuyper, M., & Hanssens, I. (1982) *Biochemistry 21*, 3594–3598.

DeKruijff, B., Demel, R. A., & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 255*, 331–347.

Estep, T. N., Mountcastle, D. B., Biltonen, R. L., & Thompson, T. E. (1978) *Biochemistry 17*, 1984–1989.

Folch, J., Lees, M., & Sloane-Stanley, C. H. (1957) *J. Biol. Chem. 226*, 497–509.

Freire, E., Markello, T., Rigell, C., & Holloway, P. W. (1983) *Biochemistry 22*, 1675–1680.

Galla, H. J., & Sackmann, E. (1974) *Biochim. Biophys. Acta 339*, 103–115.

Galla, H. J., & Hartmann, W. (1980) *Chem. Phys. Lipids 27*, 199–219.

Galla, H. J., & Luisetti, J. (1980) *Biochim. Biophys. Acta 596*, 108–117.

Galla, H. J., Theilen, U., & Hartmann, W. (1979) *Chem. Phys. Lipids 23*, 239–251.

Hartmann, W., & Galla, H. J. (1978) *Biochim. Biophys. Acta 509*, 474–490.

Hoekstra, D. (1982) *Biochemistry 21*, 1055–1061.

Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14*, 152–161.

Lakowicz, J. R. (1981) in *Spectroscopy in Biochemistry* (Bell, J. E., Ed.) Vol. 1, pp 195–245, CRC Press, Boca Raton, FL.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marinetti, G. V. (1962) *J. Lipid Res. 3*, 1–20.

Mason, J. T., Broccoli, A. V., & Huang, C. H. (1981) *Anal. Biochem. 113*, 96–101.

Newcomb, W. W., Tobin, G. J., McGowan, J. J., & Brown, J. C. (1982) *J. Virol. 41*, 1055–1062.

Pal, R., Barenholz, Y., & Wagner, R. R. (1981) *Biochemistry 20*, 530–539.

Pal, R., Wiener, J. R., Barenholz, Y., & Wagner, R. R. (1983) *Biochemistry 22*, 3624–3630.

Patzer, E. J., Moore, N. F., Barenholz, Y., Shaw, J. M., & Wagner, R. R. (1978) *J. Biol. Chem. 253*, 4544–4550.

Patzer, E. J., Wagner, R. R., & Dubovi, E. J. (1979) *CRC Crit. Rev. Biochem. 6*, 165–217.

Petri, W. A., Jr., & Wagner, R. R. (1979) *J. Biol. Chem. 254*, 4313–4316.

Petri, W. A., Jr., Estep, T. N., Pal, R., Thompson, T. E., Biltonen, R. L., & Wagner, R. R. (1980) *Biochemistry 19*, 3088–3091.

Petri, W. A., Jr., Pal, R., Barenholz, Y., & Wagner, R. R. (1981) *Biochemistry 20*, 2796–2800.

Pownall, H. J., & Smith, L. C. (1973) *J. Am. Chem. Soc. 95*, 3136-3140.
Rintoul, D. A., Chou, S. M., & Silbert, D. F. (1979) *J. Biol. Chem. 254*, 10070-10077.
Rose, J. K., & Gallione, C. J. (1981) *J. Virol. 39*, 519-528.
Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439-444.
Schloemer, R. H., & Wagner, R. R. (1975) *J. Virol. 16*, 237-249.
Selinger, Z., & Lapidot, Y. (1966) *J. Lipid Res. 7*, 174-175.
Silvus, J. R. (1982) in *Lipid-Protein Interactions* (Jost, P., & Griffith, O. M., Eds.) Vol. 2, p 239, Wiley, New York.
Verkleij, A. J., DeKruijff, B., Ververgaert, P. H., Tocanne, J. F., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 339*, 432-437.
Wagner, R. R. (1975) *Comp. Virol. 4*, 1-93.
Wiener, J. R., Pal, R., Barenholz, Y., & Wagner, R. R. (1983a) *Biochemistry 22*, 2162-2170.
Wiener, J. R., Wagner, R. R., & Freire, E. (1983b) *Biochemistry 22*, 6117-6123.
Zakowski, J. J., & Wagner, R. R. (1980) *J. Virol. 36*, 93-102.
Zakowski, J. J., Petri, W. A., Jr., & Wagner, R. R. (1981) *Biochemistry 20*, 3902-3907.

Hydrogen Exchange of Individual Amide Protons in the F Helix of Cyanometmyoglobin†

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Received May 3, 1985

ABSTRACT: Hydrogen exchange of the individual amide protons of alanine-90 (F5), glutamine-91 (F6), serine-92 (F7), and histidine-93 (F8) residues in cyanometmyoglobin of sperm whale has been studied by ^{1}H nuclear magnetic resonance spectroscopy at 360 MHz. The amide proton resonances of F5, F6, and F7 have been assigned by use of the selective nuclear Overhauser effect between the consecutive amide protons. At pH 6.8, and in the temperature range of 5-20 °C, these protons show a 10^4-fold retardation compared to the rates in free peptides. Apparent activation enthalpies for hydrogen exchange of F5, F6, and F8 protons are 18.5 ± 0.4, 9.5 ± 0.3, and 18.5 ± 0.3 kcal/mol, respectively. Some implications of these results on the nature of the opening processes involved in hydrogen exchange are considered.

Hydrogen exchange (HX)[1] of peptide NH protons with solvent protons (Hvidt & Nielsen, 1966; Woodward & Hilton, 1979; Englander & Kallenbach, 1984) has provided a powerful technique to investigate protein folding (Brems & Baldwin, 1984; Kuwajima et al., 1984), dynamics (Hilton & Woodward, 1978; Wagner & Wuthrich, 1979a; Wuthrich & Wagner, 1979), and also functional labeling or allosteric effects (Englander & Englander, 1983; Englander et al., 1983). Linderstrom-Lang first attempted to correlate hydrogen exchange behavior with protein dynamics at a time when little was known about the three-dimensional structure of proteins (Linderstrom-Lang & Schellman, 1959). The detailed mechanism of amide proton exchange in proteins still remains elusive and has been a controversial subject in contemporary biophysical chemistry (Englander & Kallenbach, 1984). Two extreme models—local unfolding (or breathing) and solvent penetration—have been proposed to account for the observed HX behavior of proteins. In the local unfolding model, the protein is assumed to undergo a transient cooperative structural unfolding with the exchange event occurring in a medium much like the bulk solvent. In solvent penetration models, on the other hand, the exchange is limited by the accessibility of each individual site to the solvent. In this case, the exchange may take place in the interior of the protein. A detailed understanding of the mechanism is essential to exploit the potential of the HX technique to probe protein structure, dynamics, and function.

In the present study, we report the HX behavior of four consecutive amide protons within the F helix of myoglobin as measured by NMR spectroscopy directly. Application of NMR spectroscopy for measuring exchange offers the advantage over other solution techniques that the rates can be measured for individual amide proton sites (Hilton & Woodward, 1978; Richarz et al., 1979; Wagner et al., 1984). Assignment of the resonances is a prerequisite for this approach to succeed. In general, the problem of NMR spectral assignments becomes increasingly difficult as the molecular size increases. Large numbers of signals, limited dispersion in chemical shifts, and relatively broad signals due to slow tumbling of macromolecules all result in loss of resolution. Assignment of the exchangeable protons in H_2O is further complicated by the dynamic range problem due to the very intense solvent peak (Redfield et al., 1975). The fact that only one amide NH proton (His F8) in sperm whale myoglobin (153 residues) has been assigned so far reflects some of these difficulties. In the present study, we have assigned three more consecutive amide protons on the basis of NH-NH-type NOEs. The hydrogen exchange behavior of consecutive protons in the F helix provides useful clues about protein flexibility as well as the fundamental mechanism of HX in these secondary structures. Myoglobin has been chosen as a model system for two reasons. First, dynamics are intrinsically relevant to function in this protein since there is no static channel available in the molecule for oxygen to reach the heme group (Case & Karplus, 1979). Second, its structure in deoxy

†Supported by National Institutes of Health Grant GM 31861.

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; MbCN, cyanometmyoglobin; HX, hydrogen exchange; His, histidine; Ser, serine; Gln, glutamine; Pro, proline; FID, free induction decay.

0006-2960/85/0424-7658$01.50/0